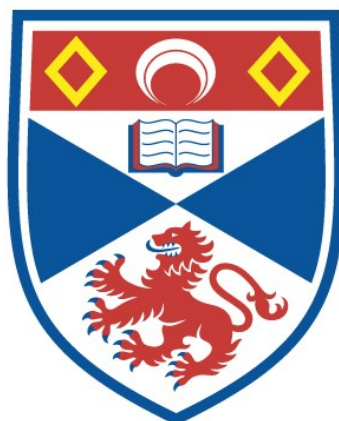


# BIOCHEMISTRY, GENETICS AND MOLECULAR BIOLOGY OF NITRITE REDUCTION IN BARLEY

Michael Patrick Ward

A Thesis Submitted for the Degree of PhD  
at the  
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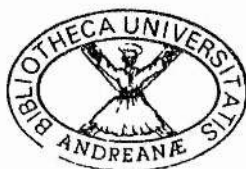
by

MICHAEL PATRICK WARD

A thesis submitted to the University of St Andrews in application for the  
degree of Doctor of Philosophy

September 1996

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## ABSTRACT

Nitrite reduction is the third step of the nitrate assimilation pathway in higher plants and is catalysed by nitrite reductase.

The whole-plant barley mutants STA1010, STA2760 and STA4169 accumulate nitrite in the leaf after treatment with nitrate and, like the *nir1* mutant STA3999 (Duncanson *et al*, 1993), lack detectable nitrite reductase cross-reacting material in the leaf and root. STA1010, STA2760 and STA4169 carry a recessive mutation in a single nuclear gene, identified as the *Nir1* locus.

RFLP analysis of the *nir1* mutant STA3999 has allowed the *Nir1* locus to be mapped to within 0.3cM of the nitrite reductase apoprotein gene, *Nii*. Studies to confirm the identity of the *Nir1* locus as *Nii*, by establishing the full-length *Nii* cDNA sequences from STA3999 and from its wild-type cv Tweed for comparative purposes, were unsuccessful as attempts to isolate a *Nii* cDNA clone from a barley cv Tweed cDNA library yielded only partial-length *Nii* clones.

These *nir1* mutants display greatly reduced nitrite reductase activity and increased NADH-nitrate reductase activity in the leaf, as compared to wild-type plants, suggesting a regulatory perturbation in the expression of the *Nar1* gene. Northern analysis shows that the *nir1* mutants possess nitrite reductase apoprotein (*nii*) transcript of wild-type size (2.3kb) and at approximately wild-type levels.

Since *nir1* mutants possess a phenotype that might be anticipated for a *Nii* mutant, it is likely that the *nir1* mutation is present in the nitrite reductase apoprotein gene *Nii* and affects translation of the *nii* transcript.

Studies of barley wild-type cv Golden Promise have demonstrated that nitrite reductase in leaf tissue is up-regulated by a coaction of nitrate and light which acts, at least partly, at the transcriptional level.

## DECLARATION

I, Michael Patrick Ward, hereby certify that this thesis, which is approximately 57,000 words in length, has been written by me, that it is a record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

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## ACKNOWLEDGEMENTS

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## ABBREVIATIONS

cv	cultivar
M <sub>1</sub>	population treated with chemical mutagen
M <sub>2</sub>	generation derived from self-pollination of M <sub>1</sub> population
M <sub>3</sub>	generation derived from self-pollination of M <sub>2</sub> population
M <sub>4</sub>	generation derived from self-pollination of M <sub>3</sub> population
M <sub>5</sub>	generation derived from self-pollination of M <sub>4</sub> population
F <sub>1</sub>	population derived from cross-pollinations between selected M population mutants and wild-type plants
F <sub>2</sub>	generation derived from self-pollination of F <sub>1</sub> population
F <sub>3</sub>	generation derived from self-pollination of F <sub>2</sub> population
F <sub>4</sub>	generation derived from self-pollination of F <sub>3</sub> population
F <sub>5</sub>	generation derived from self-pollination of F <sub>4</sub> population
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
CAT	chloramphenicol-resistance gene
CRM	cross-reacting material
CTAB	cetyltrimethylammonium bromide
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid

DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
NaFe EDTA	ethylenediaminetetra-acetic acid, ferric monosodium salt
Na <sub>2</sub> EDTA	ethylenediaminetetra-acetic acid, disodium salt
FAD	flavin adenine dinucleotide
GUS	$\beta$ -glucuronidase gene
IAA	isoamyl alcohol
LDH	lactate dehydrogenase
MES	2-[N-morpholino]ethanesulphonic acid
MOPS	3-[N-morpholino]propanesulphonic acid
NADH	$\beta$ -nicotinamide adenine dinucleotide, reduced form
NADPH	$\beta$ -nicotinamide adenine dinucleotide phosphate, reduced form
NBT	nitroblue tetrazolium
NED	N-1-naphthylethylenediamine dihydrochloride
NiR	nitrite reductase
NR	nitrate reductase
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pfu	plaque-forming units
PMS	phenazine methosulphate
PVP	polyvinylpyrrolidone
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
Szechrome NAS®	diphenyl sulphonic acid chromogen
TAE	tris-acetate buffer
TBE	tris-borate buffer
TE	tris-EDTA buffer

TEMED	N,N,N',N'-tetramethyl-ethylene diamine ethane
v/v	volume:volume
w/v	weight:volume

## CHAPTER 1

### Introduction

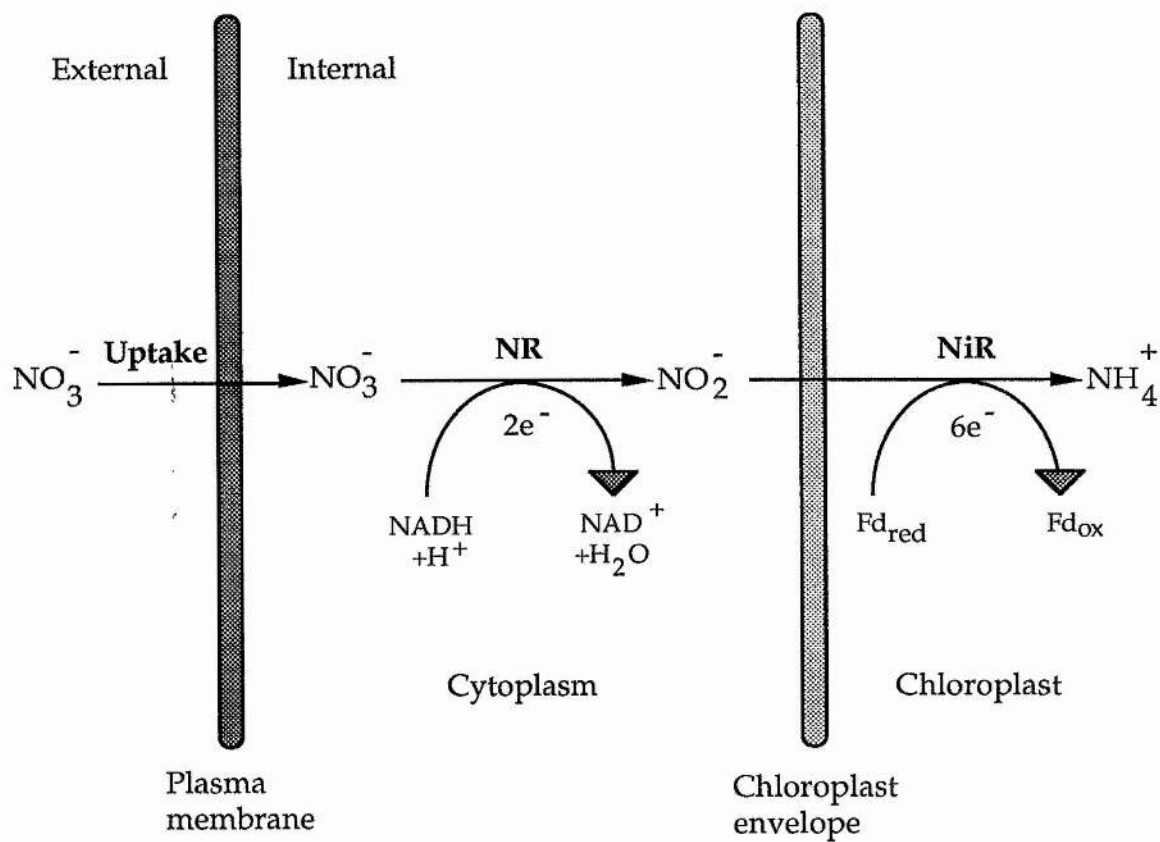
## 1.1 NITRATE ASSIMILATION - AN OVERVIEW

Nitrate is the major nitrogen source for most cultivated crop plants under normal field conditions. Although in natural ecosystems ammonium, as well as nitrate, is formed by mineralisation of organic soil nitrogen and there is considerable input of ammonium fertiliser in agricultural ecosystems, the oxidation of ammonium by autotrophic nitrifying bacteria such as *Nitrosomonas* and *Nitrobacter* ensures that in most well-aerated soils inorganic nitrogen is mainly available to the plant as nitrate (reviewed in Haynes, 1986).

Nitrogen and carbon constitute approximately 2% and 40% respectively of the dry weight of plant material. On a world-wide scale  $200 \times 10^9$  tons of carbon are fixed annually (Galston, 1961) which would necessitate the incorporation of approximately  $10 \times 10^9$  tons of nitrogen per year. Biological dinitrogen fixation in the biosphere has been estimated at  $0.175 \times 10^9$  tons per year (Burns and Hardy, 1975) and accounts for only a small proportion of total nitrogen incorporation. Thus, the majority of nitrogen incorporated into plant material occurs through the production and subsequent assimilation of nitrate.

Nitrate is converted into reduced nitrogen by the nitrate assimilation pathway (Figure 1.1) which has been studied intensively for the last 40 years due to its fundamental role in plant nutrition. Nitrate is actively taken up into the root cells where it is either stored in the vacuole, reduced in the cytoplasm or transported into, and reduced in, the leaves. Reduction of nitrate to ammonium takes place in two steps; the first step involves the reduction of nitrate to nitrite, which requires two electrons, by nitrate reductase (NR) and the second step involves the reduction of nitrite to ammonium by nitrite reductase (NiR) and requires six electrons.

Higher plants utilise, on average, about 25% of the energy required for the fixation of carbon dioxide on the assimilation of nitrate to ammonium (Guerrero *et al*, 1981) which must then be incorporated into organic compounds via the glutamine synthetase/glutamate synthase (GS/GOGAT). Thus the incorporation of nitrogen into organic compounds requires a massive amount of energy.



**Figure 1.1:** The nitrate assimilation pathway in higher plants.

Abbreviations: NR, nitrate reductase; NiR, nitrite reductase.



## 1.2 THE ENZYMES OF NITRATE ASSIMILATION

### 1.2.1 Nitrate uptake

Uptake of nitrate (Figure 1.1), its subsequent allocation to reduction, transport or storage and then remobilisation within the cell are the most poorly characterised steps of the nitrate assimilation pathway in higher plants. Recent studies have taken a more molecular approach in an effort to gain a greater understanding of these mechanisms.

#### 1.2.1.1 *Kinetics of nitrate uptake*

Nitrate ions enter the root cells against a potential gradient across the plasma membrane ranging from 70-250mV depending on tissue and species (reviewed in Larson and Ingmarsson, 1989). This suggests the existence of an active uptake system, probably in the plasma membrane. Inhibition of nitrate uptake by anaerobic conditions (Trought and Drew, 1981), uncouplers of oxidative phosphorylation (Rao and Rains, 1976) and low temperature (Clarkson and Warner, 1979) provides further evidence that metabolically-generated energy is necessary for nitrate uptake. Recent studies on tobacco cell cultures have shown that nitrate uptake depends upon membrane depolarisation, involves protein components with a short half-life and requires continuous protein synthesis (Guy and Heimer, 1993), supporting the view that nitrate uptake is via an active transport system mediated by a carrier protein.

Studies on the concentration kinetics of net ion uptake indicate the existence of at least two uptake system components, operating at high or low external nitrate concentrations respectively (Rao and Rains, 1976; Doddema and Telkamp, 1979). One system is likely to be a high-affinity "constitutive"

system and operate in plants not exposed to nitrate while the other is likely to be a low-affinity "inducible" system, characterised kinetically by studies which show that maximal rates of net nitrate uptake occur after a lag of between one and several hours after exposure of roots to nitrate (Doddema and Telkamp, 1979; Jackson *et al*, 1986; Hole *et al*, 1990; Glass *et al*, 1992). Studies in barley have also suggested that the presence of nitrate in the roots via the "constitutive" uptake mechanism is the inducer for the "inducible" mechanism rather than external nitrate (Behl *et al*, 1988). Both systems cause depolarization of the electrical potential across the plasma membrane, indicating that nitrate uptake is mediated by a nitrate/cation symport where two or more protons are co-transported into the cell with every nitrate ion (McClure *et al*, 1990; Ruiz-Cristin and Briskin, 1991; Glass *et al*, 1992).

#### 1.2.1.2 Intracellular nitrate transport

As well as the active nitrate uptake system in the plasma membrane there may also be an active carrier-mediated nitrate transport system in the tonoplast, as considerable amounts of nitrate can be accumulated in root vacuoles against a concentration gradient (Martinoia *et al*, 1981, 1986; Granstedt and Huffaker, 1982). Several tonoplast and plasma membrane proteins that appear to be nitrate inducible and hence may be involved in nitrate uptake or intracellular transport have been identified (McClure *et al*, 1987; Dhugga *et al*, 1988), including a protein in the plasma membrane which appears to be involved in the loading of nitrate into the xylem (Jackson *et al*, 1986). It has not as yet, however, been possible to confirm that these proteins are involved in nitrate uptake or transport. The fact that the nitrate reductase enzyme has been localised to several subcellular compartments, such as cytoplasmic vesicles in soybean (Vaughn and Duke, 1981; Vaughn *et al*, 1984) and chloroplasts in spinach (Kamachi *et al*, 1987), suggests that there may be

several intracellular transport systems. Wherever nitrate reductase is separated from the cytoplasm by a membrane then yet another transmembrane nitrate transport system may be necessary.

### 1.2.2 Nitrate reductase

Nitrate reduction, the second step of the nitrate assimilation pathway in higher plants (Figure 1.1), is undoubtedly the best characterised step at the physiological, biochemical and molecular level and has been the subject of several recent reviews (for example Wray, 1988; Campbell, 1988; Solomonson and Barber, 1990; Rouzé and Caboche, 1992; Campbell 1996).

Higher plant nitrate reductases are flavohaemomolybdoproteins catalysing the two electron reduction of nitrate to nitrite (Hewitt and Notton, 1980). Attempts to purify the native nitrate reductase enzyme have proven difficult due to its sensitivity to proteolytic modification (Brown *et al*, 1981; Wray and Kirk, 1981; Campbell and Wray, 1983). The native nitrate reductase enzyme is a homodimer whose subunit size, depending on species, ranges from 100-120kDa as estimated by SDS-PAGE (reviewed in Solomonson and Barber, 1990). Estimation of molecular weights of the holoenzyme vary from about 200kDa for spinach (Notton and Hewitt, 1979), tobacco (Mendel and Muller, 1980) and barley (Small and Wray, 1980) through 220-230kDa for barley (Kuo *et al*, 1980) and squash (Redinbaugh and Campbell, 1985) to 270kDa for spinach (Nakagawa *et al*, 1985).

#### 1.2.2.1 Electron donors

Two isoforms of nitrate reductase, which can use different electron donors, have been found in higher plants. The most common isoform is NADH-specific nitrate reductase (EC 1.6.6.1) which uses NADH as the

electron donor. The other isoform is an NAD(P)H-bispecific nitrate reductase (EC 1.6.6.2) which can use either NADH or NADPH as the electron donor and has been identified in several plant species occurring either as a second (low level) isoform along with the NADH-specific nitrate reductase, as in maize, barley, rice and soybean (Redinbaugh and Campbell, 1981; Streit *et al*, 1987; Kleinhoffs and Warner, 1990), or as the sole isoform, as in birch (Friemann *et al*, 1991). The reaction kinetics of nitrate reduction differ for the two higher plant isoforms. NADH-specific nitrate reductase has a pH optimum of 7.4 and a Michaelis-Menton constant ( $K_m$ ) for nitrate and NADH of 200 $\mu$ M and 2 $\mu$ M respectively. NAD(P)H-bispecific nitrate reductase, however, possesses a lower pH optimum (6.5) and a higher  $K_m$  for nitrate (4mM). The two forms can be separated by chromatography on blue dextran sepharose (Redinbaugh and Campbell, 1981) and show different developmental (Orihuel-Iranzo and Campbell, 1980) and induction (Shen *et al*, 1976) patterns. The majority of research has been carried out on NADH-specific nitrate reductase and is outlined below.

#### 1.2.2.2 Prosthetic groups

Nitrate reductase contains flavin adenine dinucleotide (FAD) (Hewitt and Notton, 1980; Redinbaugh and Campbell, 1985) and the activity of nitrate reductase is increased by the addition of exogenous FAD (Maretski *et al*, 1967; Schrader *et al*, 1968) suggesting that the flavin is readily dissociable. Purified nitrate reductase from spinach (Notton *et al*, 1977), tobacco (Mendel and Muller, 1980), barley (Somers *et al*, 1982) and squash (Redinbaugh and Campbell, 1985) have spectra indicative of the presence of a b-type cytochrome (cytochrome b<sub>557</sub>). Nitrate reductase also contains molybdenum (Mo) and the first definitive evidence for its presence was obtained with the spinach enzyme (Notton and Hewitt, 1971), although it had been previously

demonstrated in barley that a non-functional form of nitrate reductase was synthesised in the presence of the molybdenum analogue, tungsten (Wray and Filner, 1970).

Prosthetic group stoichiometry in squash suggests the presence of one FAD, one haem and one molybdenum per 115kDa nitrate reductase subunit (Redinbaugh and Campbell, 1985). Molybdenum is carried as a molybdenum cofactor (MoCo) which is a complex between molybdenum and a phosphorylated pterin, molybdopterin (Johnson and Rajagopalan, 1982). The pterin acts as a chelator of molybdenum, interfacing it to the protein thus conferring biological activity upon it and is also responsible for the dimerisation of the protein subunits (Ketchum *et al*, 1970). The electron pathway in nitrate reduction has been established as NADH-[FAD-haem-molybdenum]-nitrate (reviewed in Hewitt and Notton, 1980).

#### 1.2.2.3 *Partial activities associated with the nitrate reductase holoenzyme*

In addition to the physiological reduction of nitrate with NADH as the electron donor, nitrate reductase can also catalyse several partial activities *in vitro* involving one or more of the prosthetic groups and using artificial electron donors or acceptors (reviewed in Wray and Fido, 1989; Hoff *et al*, 1994). The partial activities are classified as NADH dehydrogenase (diaphorase) activities or as terminal nitrate reductase activities. The dehydrogenase activity is associated with the proximal part of the electron transport chain, perhaps FAD (Brown *et al*, 1981), uses NADH as the electron donor and reduces a variety of artificial electron acceptors such as ferricyanide and cytochrome c. The terminal nitrate reductase activities are molybdenum-dependant, hence are associated with the distal part of the electron transport chain, and involve the reduction of nitrate to nitrite by

artificial electron donors such as reduced methyl viologen, reduced bromophenol blue and flavin nucleotides.

#### 1.2.2.4 Location

Most available evidence points to a cytosolic location of nitrate reductase (reviewed in Solomonson and Barber, 1990) although it has been proposed that the enzyme is present within peroxisomes (Lips and Avissar, 1972), cytoplasmic vesicles (Vaughn and Duke, 1981; Vaughn *et al*, 1984) and chloroplasts (Kamachi *et al*, 1987). A membrane-bound form of nitrate reductase has also been suggested (Ward *et al*, 1988, 1989; Hoarau *et al*, 1991; Stohr *et al*, 1993).

#### 1.2.3 Nitrite reductase

The six-electron reduction of nitrite to ammonium ions is the third step of the nitrate assimilation pathway in higher plants (Figure 1.1) and is catalysed by ferredoxin:nitrite oxidoreductase (EC 1.7.7.1) (nitrite reductase). This step was first identified thirty-five years ago when Huzisige and Satoh (1961) demonstrated that light stimulated the disappearance of nitrite from solution in the presence of chloroplast grana and a soluble fraction from spinach leaves. They ascribed this to a photochemical reduction process. The presence of nitrite reductase activity in higher plants was established by Hageman *et al* (1962) who demonstrated the stoichiometric conversion of nitrite to ammonium ions when marrow leaf extracts were incubated with reduced benzyl viologen dye. Subsequently, reduced ferredoxin was shown to be the natural electron donor in the photochemical reduction of nitrite (Paneque *et al*, 1963; Losada *et al*, 1963; Huzisige *et al*, 1963). Nitrite reductase



activity has also been demonstrated in non-chlorophyllous tissue, such as root tissue, and is described later in this chapter.

Nitrite reductase has been purified to apparent homogeneity from the leaves of a number of species including spinach (Ho and Tamura, 1973; Ida and Morita, 1973; Ida *et al*, 1976; Vega and Kamin, 1977; Ida, 1977; Hirasawa and Tamura, 1980; Ida and Mikami, 1986), *Cucurbita pepo* (Hucklesby *et al*, 1976), barley (Serra *et al*, 1982; Ip *et al*, 1990), wheat (Small and Gray, 1984) and *Phaseolus angularis* (Ishiyama and Tamura, 1985; Ishiyama *et al*, 1985). The enzymes are usually isolated as monomeric polypeptides of 60-63kDa. However, Tamura and coworkers (Hirasawa and Tamura, 1980; Hirasawa-Soga and Tamura, 1981; Hirasawa *et al*, 1982) reported a molecular weight of around 86kDa for a spinach nitrite reductase which separates into two components of 61kDa and 24kDa after DEAE-Sephadex A-50 chromatography. Hirasawa-Soga *et al* (1982) speculated that the larger component (61kDa) is a modified form of the native enzyme having only methyl viologen-linked activity whilst the smaller component acts as a "coupling" protein which confers ferredoxin-linked activity. However, these results appear to disagree with the observation that purified spinach nitrite reductase of molecular weight 63kDa can utilise ferredoxin and methyl viologen equally effectively as an electron donor (Joy and Hageman, 1966; Ida and Mikami, 1986).

The amino acid composition of purified nitrite reductase protein has been determined in spinach (Vega and Kamin, 1977; Ida and Mikami, 1986), *Cucurbita pepo* (Hucklesby *et al*, 1976) and barley (Ip *et al*, 1990) producing nearest integer values of 564, 558, 565 and 575 amino acid residues respectively. The calculated molecular weight of leaf nitrite reductase from the amino acid composition lies within the range 60-63kDa while the spinach apoprotein gene encodes a mature protein of molecular weight 63kDa (Back *et al*, 1988). The recent characterisation of nitrite reductase apoprotein genes

from other species has allowed further understanding of the nitrite reductase enzyme and is described later in this chapter.

The pH optimum of the nitrite reductase enzyme varies, with an optimum of 7.5 for the spinach leaf form (Ida and Morita, 1973) and a lower optimum of pH 6.0-6.5 reported for the barley leaf form (Serra *et al*, 1982). However, this low pH is surprising as the import of nitrate ions into the cell will cause the intracellular pH to rise above 7.0. The enzyme also possesses a low thermostability (Ho and Tamura, 1973; Serra *et al*, 1982) with enzyme activity being almost completely destroyed after treatment at 60°C for 5 minutes (Ho and Tamura, 1973).

Isoforms of nitrite reductase have been identified in several species. Two forms are present in the root, scutellum and etiolated shoots of maize (Hucklesby *et al*, 1972; Dalling *et al*, 1973) and in the roots of wheat (Dalling *et al*, 1972b). Isoforms have also been found in pea and tomato (Kutscherra *et al*, 1987). Two of the three isoforms found in wild oat are encoded by different genetic loci, confirming their identity as isoenzymes (Heath-Pagliuso *et al*, 1984), but the relationship between the isoforms of other species is less clear. Isoforms are generally of the same size with similar kinetic properties but differences in thermal stability have been noted (Hucklesby *et al*, 1972). Differences in the timing of development of isoforms have been identified in wheat with only the main isoform being dependant on light and nitrate for development (Kutscherra *et al*, 1987).

#### 1.2.3.1 *Electron donors*

As described previously, ferredoxin is widely accepted as the physiological electron donor in leaf tissue, reduced *in vivo* by electron transport associated with photosystem I (Neyra and Hageman, 1974) and *in vitro* by dithionite (Joy and Hageman, 1966). Nitrite reductase can also utilise



reduced viologen dyes (methyl and benzyl viologen) as electron donors (Hageman *et al*, 1962; Joy and Hageman, 1966) for *in vitro* nitrite reduction but is unable to use reduced pyridine nucleotides without the addition of the separate activity NADPH-ferredoxin reductase and ferredoxin (Hageman *et al*, 1962).

Purified spinach leaf nitrite reductase possesses a greater affinity for nitrite when ferredoxin is the electron donor with a  $K_m$  of  $10.3\mu\text{M}$  as compared to a  $K_m$  of  $110\mu\text{M}$  possessed by the methyl viologen-linked enzyme (Ida and Mikami, 1986). However, Serra *et al* (1982) reported a  $K_m$  value for nitrite of  $250\mu\text{M}$  obtained using either ferredoxin or methyl viologen as electron donor for barley nitrite reductase. The purified enzyme appears to possess a high affinity for ferredoxin with a  $K_m$  value of  $6\mu\text{M}$  for the spinach form (Ida and Mikami, 1986), although in earlier studies Ho and Tamura (1973) reported a  $K_m$  value for ferredoxin of  $70\mu\text{M}$ . Hirasawa-Soga and Tamura (1981) have reported a  $K_m$  value for ferredoxin of  $27\mu\text{M}$  for the 86kDa protein species purified from spinach leaves. Estimates of the  $K_m$  value for methyl viologen range from  $64\mu\text{M}$  for spinach nitrite reductase (Ida and Morita, 1973) to  $120\mu\text{M}$  for spinach (Ida and Mikami, 1986) and barley (Serra *et al*, 1982) nitrite reductase.

#### 1.2.3.2 Prosthetic groups

Nitrite reductase contains sirohaem (Murphy *et al*, 1974; Vega and Kamin, 1977), which has also been found in several higher plant sulphite reductases. Nitrite reductase sirohaem is an iron tetrahydroporphyrin of the isobacteriochlorin type (Murphy *et al*, 1974) and expresses a visible spectrum with absorption maxima in spinach nitrite reductase at 276, 386, 573 and 690nm (Vega and Kamin, 1977). This characteristic spectrum has also been found in purified nitrite reductase from other species including *Cucurbita*

*pepo* (Hucklesby *et al*, 1976), bean (Ishiyama *et al*, 1985) and barley (Serra *et al*, 1982; Ip *et al*, 1990).

Nitrite reductase also contains an iron-sulphur centre, originally thought to be a  $\text{Fe}_2\text{S}_2$  centre (Vega and Kamin, 1977) but shown later to be a tetranuclear  $\text{Fe}_4\text{S}_4$  centre (Lancaster *et al*, 1979). Haem ligands such as carbon monoxide and cyanide were found to modify both the reducibility and the electron paramagnetic resonance (EPR) signal line shape of the  $\text{Fe}_4\text{S}_4$  centre, with the addition of carbon monoxide resulting in a 10-fold increase in intensity of the reduced iron-sulphur centre EPR signal (Lancaster *et al*, 1979). This indicates an interaction between the haem and the  $\text{Fe}_4\text{S}_4$  centre of the active site, with the sirohaem moiety binding nitrite and accepting electrons from the  $\text{Fe}_4\text{S}_4$  centre which itself is reduced by ferredoxin *in vivo* or by reduced methyl viologen *in vitro*.

#### 1.2.3.3 Location

Since intact chloroplasts from a number of species can photoreduce nitrite to ammonium (Paneque *et al*, 1963) and subsequently to  $\alpha$ -amino-nitrogen (Magalhaes *et al*, 1974; Anderson and Done 1978) and the enzyme's electron donor in leaves is reduced ferredoxin, it is usually concluded that the chloroplast is the sole site of functional nitrite reductase within the leaf cell. This conclusion is supported by cell fractionation studies (for example Dalling *et al*, 1972a; Wallsgrove *et al*, 1979). Recently, however, evidence for an extra-plastidic form of nitrite reductase in the cotyledons of etiolated mustard seedlings has been presented (Schuster and Mohr, 1990b).

Initial attempts to determine which genome (nuclear or chloroplastic) encodes the nitrite reductase apoprotein depended on the use of inhibitors of protein synthesis in the cytoplasmic (80S) and the chloroplastic (70S) ribosomes, but were inconclusive (Stewart, 1968; Sluiters-Scholton, 1973).

Molecular studies (Small and Gray, 1984; Gupta and Beevers, 1985, 1987; Ogawa and Ida 1987) involving *in vitro* translation of poly A<sup>+</sup> RNA and immunoprecipitation of products with specific nitrite reductase antiserum demonstrated that, like many other nuclear-encoded chloroplast enzymes (reviewed in Archer and Keegstra, 1990), nitrite reductase is synthesised as a precursor protein with a N-terminal extension, the transit peptide, which acts to target the precursor protein to and within the chloroplast. The precursor protein can be processed in a two-step reaction to a polypeptide of the same size as that of the native protein ( $M_r$  ca. 63kDa) (Gupta and Beevers, 1987). Studies on enzyme polymorphism in oat (Heath-Pagliuso *et al*, 1984) and Mendelian inheritance of the nitrite reductase apoprotein gene in barley (Duncanson *et al*, 1993) provide further evidence that the nitrite reductase gene is nuclear-encoded.

#### 1.2.3.4 Nitrite reductase in non-chlorophyllous tissue

The existence of functional nitrite reductase has been demonstrated in non-chlorophyllous tissue such as maize scutellum, where two isoforms with similar properties to each other and to leaf nitrite reductase were identified (Hucklesby *et al*, 1972; Dalling *et al*, 1973). Nitrite reductase activity observed in the roots has been localised in the plastids (Dalling *et al*, 1972b; Miflin, 1974; Emes and Fowler, 1979; Oaks and Hirel, 1985). Nitrite reductase activity is increased in plastids isolated from root tissue of nitrate-grown pea, which also possess an increased flow of carbon through the oxidative pentose phosphate pathway, compared with root plastids from pea plants grown in the absence of nitrate (Emes and Fowler, 1983). Plastids from the root tissue of nitrate-grown barley plants also contain a glucose-6-phosphate and NADP<sup>+</sup>-linked nitrite reductase system (Oji *et al*, 1985) with both benzyl and methyl viologen enzymatically reduced by plastid extract in the

presence of glucose-6-phosphate and NADP<sup>+</sup>. The identification of a pyridine nucleotide reductase immunologically similar to spinach leaf ferredoxin-NADP<sup>+</sup> reductase (Suzuki *et al*, 1985), of a ferredoxin-like electron carrier (Ninomiya and Sato, 1984; Suzuki *et al*, 1985; Bowsher *et al*, 1993), support the suggestion that the supply of reducing power for *in vivo* root nitrite reduction originates from the oxidative pentose phosphate pathway.

Nitrite reductase protein has been purified from the root tissue of several species such as barley (Ida *et al*, 1974) and pea (Bowsher *et al*, 1988). The nitrite reductase enzyme from pea root has a molecular weight of about 60kDa and exhibits absorption maxima at 278, 384, 573 and 695nm (Bowsher *et al*, 1988) similar to spinach leaf nitrite reductase (Vega and Kamin, 1977) and indistinguishable from that of *Cucurbita pepo* leaf nitrite reductase (Bowsher *et al*, 1988), whilst reduction of the purified enzyme with dithionite in the presence of cyanide allows the appearance of an EPR signal characteristic of an iron-sulphur centre (Bowsher *et al*, 1988). The purified nitrite reductase from pea roots also cross-reacts with antibodies raised against *Cucurbita pepo* leaf nitrite reductase in Ouchterlony double-diffusion experiments (Bowsher *et al*, 1988). Nitrite reductase from leaf and root tissues produces single lines of identity in pea (Bowsher *et al*, 1988) and bean (Ishiyama *et al*, 1985) suggesting that, in these species at least, nitrite reductase is very similar in both leaf and root tissues.

## 1.3 CHARACTERISATION OF NITRATE ASSIMILATION GENES

### 1.3.1 Nitrate transport genes

Recently, several putative nitrate transporter cDNA clones have been isolated from a barley cDNA library using a partial clone, amplified from root first-strand cDNA using the polymerase chain reaction (PCR) with degenerate primers designed from the sequence of the *Aspergillus nidulans* nitrate uptake gene *crnA*, as a probe (Trueman and Forde, 1993). Identification of the clones as nitrate transporter clones has not yet been confirmed.

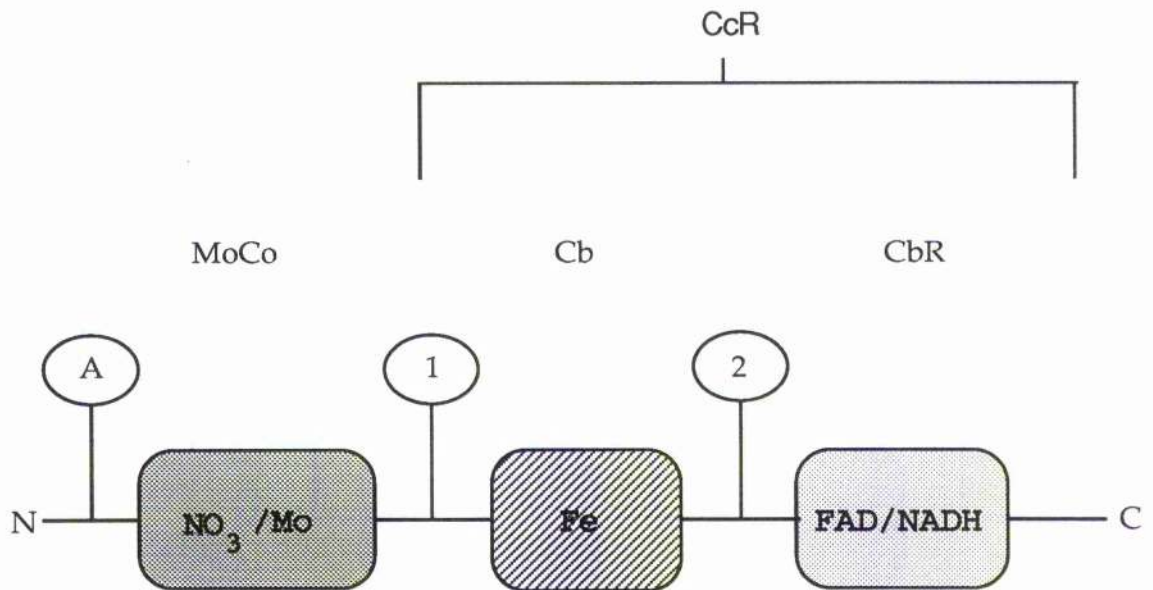
### 1.3.2 Nitrate reductase genes

The complete or partial nucleotide sequence of many higher plant nitrate reductase apoprotein (*nia*) genes or cDNA clones has been determined (reviewed in Rouzé and Caboche, 1992) and have subsequently given much insight into the structure and function of the nitrate reductase apoprotein. Studies of both the deduced amino acid sequence similarity between the nitrate reductase apoproteins of different species and the similarity of the deduced nitrate reductase apoprotein sequences to the sequences of known proteins with defined functionality has shown that the prosthetic group binding regions are laid out linearly in the apoprotein backbone (Campbell and Kinghorn, 1990; Solomonson and Barber, 1990; Rouzé and Caboche, 1992). The N-terminal region of the nitrate reductase apoprotein is similar to the MoCo binding region of the mammalian enzyme sulphite oxidase (EC 1.8.3.1), the central region is similar to mammalian and plant cytochrome b<sub>5</sub>, a haem-Fe protein, and the C-terminal region is similar to mammalian cytochrome b<sub>5</sub> reductase (EC 1.6.2.2), a FAD-containing

enzyme (Figure 1.2). In each case, the entire homologous region is contained within the nitrate reductase gene sequence without interruption which suggests that *nia* genes evolved through the fusion of introns flanking the functional domains (reviewed in Campbell, 1996).

Three further regions of amino acid sequence were found to be highly variable upon comparison of the nitrate reductase apoprotein sequences between species; a region at the N-terminus, which appears to play a role in nitrate reductase stability and light regulation (Nassaume *et al*, 1995), and two "hinge" regions, one either side of the MoCo binding domain (Figure 1.2). Hinge 1 contains a trypsin site and hinge 2 may also contain a proteolytic site (Bright *et al*, 1981; Solomonson and Barber, 1990). The reason for the nitrate reductase enzyme having all of its components in one polypeptide is unclear, although it has been suggested that the enzyme may have evolved to simplify the regulation mechanism of nitrate reduction (Campbell, 1996).





**Figure 1.2:** Model of the higher plant nitrate reductase (NR) subunit.

The amino and carboxyl termini are labelled N and C respectively. The functional domains of NR are indicated at the top: MoCo (molybdenum cofactor); Cb (cytochrome b domain) and CbR (cytochrome b reductase) which constitute the CcR (cytochrome c reductase) domain. Within the domains themselves are shown the cofactors and substrates of nitrate reductase: NO<sub>3</sub>, nitrate-binding and reduction site; Mo, molybdate/molybdopterin-binding site; Fe, Fe-haem-binding site; FAD, FAD-binding site; NADH, NADH-binding site. Three areas of low amino acid sequence homology are circled: A, N-terminal domain; 1, hinge 1; 2, hinge 2. Adapted from Campbell (1996).

### 1.3.3 Nitrite reductase genes

The complete or partial nucleotide sequence of a number of nitrite reductase apoprotein (*nir*) genes or cDNA clones has been determined (summarised in Table 1.1). Comparisons of nitrite reductase apoprotein amino acid sequences, deduced from full length cDNA clones of different species, has allowed identification of the N-terminal transit peptide regions of the precursor protein (Back *et al*, 1988; Friemann *et al*, 1992a). Comparison of the deduced amino acid nitrite reductase apoprotein sequences with the amino acid sequences of known enzymes of defined functionality has yielded information about the functional domains within the nitrite reductase enzyme, where possible binding sites for ferredoxin (Friemann *et al*, 1992a) and the sirohaem/Fe<sub>4</sub>S<sub>4</sub> centre (Siegel and Wilkerson, 1989) have been identified. The analysis of nitrite reductase apoprotein amino acid sequence comparisons is discussed in more detail in Chapter 6.



**Table 1.1: Summary of characterised higher plant nitrite reductase clones**

Species	Clone	cDNA Length (bp)	Protein Length (aa)	Reference
Spinach	cDNA	2062	595	Back <i>et al</i> (1988)
	Genomic	4234	595	Back <i>et al</i> (1991)
Maize	cDNA*	1813	568	Lahners <i>et al</i> (1988)
Birch	cDNA	1752	584	Friemann <i>et al</i> (1992a)
Tobacco	<i>nir1</i> cDNA*	1754	584	Vaucheret <i>et al</i> (1992a)
	<i>nir2</i> cDNA*	1043	347	Kronenberger <i>et al</i> (1993)
	<i>nir3</i> cDNA*	1457	457	Kronenberger <i>et al</i> (1993)
Pine	cDNA*	321	106	Neininger <i>et al</i> (1994)
<i>Arabidopsis</i>	Genomic	4380	587	Tanaka <i>et al</i> (1994)
Rice	cDNA	2410	597	Terada <i>et al</i> (1995)
Bean	Genomic	5546	583	Sander <i>et al</i> (1995)
Barley	cDNA	503	71	Ward <i>et al</i> (1995)

\* Denotes partial sequence; cDNA length is given as number of nucleotides in base pairs (bp); protein length is given as the number of amino acid (aa) residues.

## 1.4 REGULATION OF THE NITRATE ASSIMILATION PATHWAY

### 1.4.1 Nitrate uptake

The expression of nitrate uptake genes has been studied mainly as the rate of uptake by intact plants, excised roots or other plant tissues. Nitrate uptake rate depends on several factors, such as nitrate efflux rates, root temperature and internal nitrogen demand (reviewed in Larsson and Ingmarsson, 1989), although most studies have concentrated on the relationship between uptake characteristics and variations in nitrate supply.

One of the salient features of nitrate uptake is that the ability to take up nitrate is absent or low in plants deprived of nitrate and upon readdition of nitrate the uptake system is induced and full activity is usually seen 2-10 hours later (Clarkson, 1986; Jackson *et al*, 1986). Inhibitors of mRNA transcription and translation block apparent induction of nitrate uptake (Jackson *et al*, 1973; Clarkson 1986) which suggests *de novo* synthesis of one or more polypeptides is important for nitrate uptake, although this observation may be due to an indirect effect of some other general plant response to the inhibitors on nitrate uptake. Functional NR does not have a prerequisite for induction of nitrate uptake (Doddema *et al*, 1978) and it is generally acknowledged that nitrate itself is the inducer of nitrate reductase (Jackson *et al*, 1986). Several studies have shown that the pattern of uptake induction depends on external nitrate concentration (Morgan *et al*, 1985; Mack and Tischner, 1986; Goyal and Huffaker, 1986) although this may be related to the differential response of several uptake systems.

Light may also play a role in nitrate uptake. Non-nodulated soybean plants were shown to display diurnal rhythms, with uptake being at a much reduced level during the dark period. When the dark period was interrupted by low intensity light the nitrate uptake rate increased to double the level of

uptake during the light period (Raper *et al*, 1991). Down-regulation by end-products of nitrate assimilation also appears to have a role, as nitrate uptake in maize was found to decrease when amino acids were added to the nutrient medium (Padgett and Leonard, 1993).

#### 1.4.2 Nitrate reductase

Control of nitrate reductase activity in higher plants has been studied primarily in leaf tissue, and can be effected in two ways; firstly, nitrate reductase activity levels can be raised and lowered by *de novo* nitrate reductase synthesis and nitrate reductase degradation respectively or, secondly, the mature enzyme can be inactivated and reactivated post-translationally.

##### 1.4.2.1 Transcriptional control of nitrate reductase by light and nitrate

In general, nitrate reductase activity is highest in plants grown on nitrate in the presence of light (for example, Beevers and Hageman, 1969; Gupta and Beevers 1983). While nitrate application leads to increases in nitrate reductase activity levels, it is not an obligatory requirement since considerable nitrate reductase activity levels can be found in soybean plants (Lahav *et al*, 1976) and cultured tobacco cells (Buchanan and Wray, 1982; Muller, 1983) that have never been exposed to nitrate.

Immunological studies (Somers *et al*, 1983) have shown that the increase in nitrate reductase activity seen after nitrate treatment of plants is due to *de novo* synthesis of nitrate reductase protein. Northern analysis demonstrates that the steady state level of nitrate reductase mRNA increases markedly (over 100-fold) after treatment with nitrate (Cheng *et al*, 1986; Crawford *et al*, 1986), demonstrating that nitrate acts at the transcriptional

level and that increased transcription is the underlying mechanism of *de novo* synthesis of nitrate reductase (reviewed in Solomonson and Barber, 1990; Rouzé and Caboche, 1992). However, the signals stimulating nitrate reductase degradation have not yet been identified, although it is likely that degradation begins by proteolysis at either of the hinge regions or attack at the N-terminus prior to total degradation of the protein (Bright *et al*, 1981).

Nitrate reductase activity remains at basal levels in pea leaf treated with nitrate in the dark (Gupta and Beevers, 1983), suggesting that nitrate reductase activity levels in the leaf are also influenced by the intensity of illumination (Sanderson and Cocking, 1964; Beevers *et al*, 1965). In order to study light-effected induction of nitrate reductase activity, Hageman and Flesher (1960) used etiolated maize plants either pre-treated with nitrate or not pretreated. The non-pretreated plants were then treated with nitrate and light at the same time and induction of leaf nitrate reductase activity was initially slow and only became rapid after several hours, whereas induction of nitrate reductase activity in the leaves of nitrate-pretreated plants was immediate when treated with light (Hageman and Flesher, 1960). They concluded that both nitrate and light influence nitrate reductase activity. Many subsequent studies have found that light only increases leaf nitrate reductase activity if plants have been pretreated with nitrate (Travis *et al*, 1970; Beevers and Hageman, 1972; Abrol *et al*, 1983; Gowri *et al*, 1992) and in general, it has been concluded that nitrate is required for light induction to occur. Studies of etiolated plants have shown that red light can substitute for white light in the regulation of nitrate reductase, suggesting phytochrome is responsible for light induction of leaf nitrate reductase activity (Beevers and Hageman, 1972; Srivastava, 1980; Abrol *et al*, 1983). Further studies have suggested that intracellular free  $\text{Ca}^{2+}$  is also involved in the phytochrome-mediated regulation of NR (Bergareche *et al*, 1994).

Nitrate reduction may also be controlled by an unknown "plastidic" factor, as intact plastids have been shown to be necessary for nitrate reductase enzyme activity and gene transcription in mustard (Schuster and Mohr, 1990b).

A number of experiments have shown that nitrate reductase activity can be induced in the dark by applying nitrate if the plants have adequate energy reserves to synthesise proteins (Travis *et al*, 1970; Huffaker, 1982; Abrol *et al*, 1983) although activity levels are low. Cheng *et al* (1992) found that sucrose can mimic the light induction of nitrate reductase mRNA in the leaves of *Arabidopsis*, and is sufficient for full induction of nitrate reductase activity in the presence of nitrate. Further studies in tobacco (Vincentz *et al*, 1993) show that glucose and fructose, as well as sucrose, can produce this effect.

#### 1.4.2.2 Post-translational regulation of nitrate reductase

Nitrate reductase activity can be controlled by a post-translational mechanism involving phosphorylation of the nitrate reductase protein and binding via  $Mg^{2+}$  or another divalent cation to an inhibitor protein (reviewed in Kaiser and Huber, 1994). Post-translational regulation appears to be the end result of a signal transduction pathway where the stimuli are light/dark transitions and other environmental factors affecting other major plant processes such as carbon metabolism (Campbell, 1996). The phosphorylation site of the nitrate reductase apoprotein has recently been identified as Ser<sup>543</sup> in the hinge 1 region of spinach nitrate reductase (Bachmann *et al*, 1996) and Ser<sup>534</sup>, the corresponding serine residue, in *Arabidopsis* (Su *et al*, 1996).

Studies in etiolated barley (Somers *et al*, 1983) demonstrated that when plants were deprived of nitrate, the leaf nitrate reductase activity level

decreased before the nitrate reductase protein level, suggesting a temporary post-translational inactivation of the nitrate reductase protein before protein degradation takes over. Riens and Heldt (1992) demonstrated that when nitrate-treated spinach plants are transferred from light to dark, nitrate reductase activity level decreases, with a half-life of 2 minutes, to 15% of the level in light. This light/dark modulation of nitrate reductase is extremely important as nitrate reduction, unlike nitrite reduction, does not require redox energy generated by photosystem I, hence must be switched off in order to prevent the accumulation of toxic nitrite. Similar results were observed in pea (Kaiser *et al*, 1993) and light/dark modulation in spinach and maize has been positively linked to protein phosphorylation (Huber *et al*, 1992, 1994).

#### 1.4.2.3 Down-regulation of nitrate reductase by N-metabolites

Experiments have been performed involving glutamine synthetase (GS) inactivation (Deng *et al*, 1991) in tobacco which demonstrates a sharp decrease in glutamine levels and results in accumulation of nitrate reductase mRNA, and glutamine treatment of spinach cell culture (Shiraishi *et al*, 1992) and *N. plumbaginifolia* leaves (Vincentz *et al*, 1993) which causes a decrease in nitrate reductase activity and mRNA. These results suggest a negative feedback mechanism acting on the nitrate assimilation pathway with the effector being an N-metabolite, probably glutamine.

#### 1.4.2.4 Circadian rhythms of nitrate reductase

Nitrate reductase mRNA of leaves or shoots of plants grown in a day/night cycle fluctuate with a maximal level at the beginning of the day or a few hours after the onset of light (for example in tomato and tobacco



(Galangau *et al*, 1988; Deng *et al*, 1990; Becker *et al*, 1992), maize (Bowsher *et al*, 1991) and *Arabidopsis* (Cheng *et al*, 1991)) and, where studied, these fluctuations also affect NR protein and activity in the same way. In contrast, nitrate reductase mRNA does not appear to fluctuate in roots (Bowsher *et al*, 1991; Deng *et al*, 1991). The cyclic fluctuations of nitrate reductase mRNA may be due to the down-regulation of transcription by glutamine, as glutamine pools seem to fluctuate in the opposite phase (Deng *et al*, 1991). Whether circadian rhythm affects transcription or mRNA stability is unclear. In *N. plumbaginifolia*, the circadian rhythm of nitrate reductase mRNA appears to result from transcriptional control (Vincentz and Caboche, 1991; Vaucheret *et al*, 1992b). However in *Arabidopsis*, nuclear run-on experiments led to the conclusion that nitrate reductase mRNA oscillations are due to post-transcriptional regulation (Pilgrim *et al*, 1993).

### 1.4.3 Nitrite reductase

#### 1.4.3.1 Regulation of nitrite reductase in etiolated plants

Nitrate and light, as well as an unidentified plastidic factor produced by functional plastids, are required for the formation of nitrite reductase activity (reviewed in Mohr *et al*, 1992). The light requirement operates via phytochrome, but the nature of the interaction between these effectors and the method of regulation appears to differ between plant species. In a comparative study between mustard, spinach, tobacco and barley it was shown that in etiolated mustard seedling cotyledons a strong synergism existed between nitrate and light with respect to enzyme synthesis, but that the nitrite reductase mRNA level was determined by light (Schuster and Mohr, 1990a). In etiolated spinach seedling cotyledons the action of light on nitrite reductase activity level was superimposed multiplicatively on the

action of nitrate, indicating that each factor acts independently, but transcript level was determined by nitrate (Seith *et al*, 1991). In contrast, in etiolated tobacco seedling cotyledons (Neininger *et al*, 1992) and in etiolated barley shoots (Seith 1994) no light effect on enzyme activity was seen in the absence of nitrate, and a coaction of light and nitrate was required for a high nitrite reductase mRNA level. However, regardless of species, the plastidic factor is a prerequisite for nitrate and/or light action.

#### 1.4.3.2 Regulation of nitrite reductase in green, white-light-grown plants

Up-regulation of nitrite reductase transcript level and activity in leaves of green, white-light-grown plants is controlled by a coaction of light and nitrate (spinach, Back *et al*. 1988; maize, Bowsher *et al*, 1991; Lahners *et al*, 1988; *N. plumbaginifolia*, Faure *et al*, 1991; birch, Friemann *et al*, 1992b; Scots pine, Neininger *et al*, 1994). How light operates to control transcript level is unclear but it is unlikely to act directly and attempts to demonstrate a role for phytochrome have been inconclusive. Becker *et al* (1992) studied the light-regulated expression of the nitrate reductase and nitrite reductase genes in the leaves of the phytochrome-deficient *aurea* mutant of tomato. The *aurea* mutant contains no more than 5% of the wild-type level of phytochrome. The studies suggested that either phytochrome was not involved or that the reduced amount of phytochrome present does not limit expression.

The observation that the decrease in leaf nitrate reductase activity which results when green, white-light-grown plants are transferred to the dark can be reversed by exogenously supplied sugars suggests that light may indirectly affect nitrate assimilation via carbohydrates synthesised through photosynthesis. As described previously, it has been demonstrated that carbohydrates such as glucose, sucrose and fructose (Cheng *et al*, 1992; Vincentz *et al* 1993) can replace light in eliciting an increase in nitrate



reductase activity and/or mRNA accumulation in higher plants. In the case of nitrite reductase, however, neither transcript level or activity, nor *rbcS* transcript level could be increased by feeding glucose in the dark to leaves of *N. plumbaginifolia* detached from 56 hour dark-adapted, nitrate-grown plants (Vincentz *et al*, 1993). Vincentz *et al* (1993) also showed that similar leaf nitrate reductase transcript levels occur at both high and low light intensities, whereas leaf nitrite reductase and *rbcS* transcript levels were higher at high light intensity. This suggests that the light regulation of nitrite reductase genes is related more closely to that of photosynthetic genes than to that of the nitrate reductase gene.

Down-regulation of nitrite reductase, like that of nitrate reductase, is probably controlled by nitrogenous end-products of nitrate assimilation, and several studies have provided results to support this hypothesis. In nitrate reductase-deficient mutants of *N. plumbaginifolia*, both nitrite reductase and nitrate reductase transcripts are elevated (Pouteau *et al*, 1989; Faure *et al*, 1991). Feeding glutamine, glutamate or asparagine to detached leaves of these *N. plumbaginifolia* mutants at low light intensities decreased the nitrite reductase transcript level, though not to the same extent as for nitrate reductase (Vincentz *et al*, 1993). In addition, circadian fluctuations of nitrate reductase and nitrite reductase transcript levels, being high at the end of the night period and decreasing during the succeeding light period, correlate inversely with leaf glutamine concentration (Faure *et al*, 1991; Bowsher *et al*, 1991; Deng *et al*, 1991).

The regulation of nitrite reductase in green, white-light-grown plants is described in further detail in Chapter 3.

The regulation of nitrite reductase in roots is not so well characterised. In roots of green, white-light-grown nitrate-less maize plants, nitrite reductase transcript levels increase on addition of nitrate, and decline on its withdrawal with a half life of less than 30 minutes (Lahners *et al*, 1988;

Kramer *et al*, 1989). Studies suggest that the synthesis of leaf and root nitrite reductase is regulated differently in barley (Duncanson *et al*, 1992). Both nitrate and light are required for enzyme production in the leaf, but nitrate alone is sufficient for induction of nitrite reductase synthesis in the root. Since there only appears to be a single nitrite reductase apoprotein gene in barley (Duncanson *et al*, 1993), the regulatory differences observed between leaf and root are most probably located in the pathway that transduces the environmental signals, nitrate and light, and are not due to differences that might exist between the *cis*-acting DNA regulatory elements of a leaf-active nitrite reductase apoprotein gene or a different, root-active nitrite reductase apoprotein gene.

## 1.5 NITRATE ASSIMILATION MUTANTS

The first higher plants affected in the nitrate assimilation pathway were isolated in *Arabidopsis* (Oostinder-Braaksma and Feenstra, 1973) when a putative nitrate uptake mutant was selected. Since then, mutants in this pathway have been isolated in a number of different species (reviewed in Hoff *et al*, 1994), most of which are affected in nitrate reductase activity. Several agents have been used as mutagens, such as sodium azide, MNNG (N-methyl-N'-nitro-N-nitrosoguanidine), EMS (ethyl methane sulphonate), and UV and gamma irradiation either at the seed level or in plant tissue culture (reviewed in Hoff *et al*, 1994). In addition, spontaneous mutations have arisen in cell culture, some of which were the result of transposon insertions by endogenous transposons (Grandbastien *et al*, 1989; Vaucheret *et al*, 1992b; Tsay *et al*, 1993a; Meyer *et al*, 1994).

Three methods for selection of mutants affected in nitrate assimilation have been routinely used. The predominant method has been on the basis of chlorate resistance (reviewed in Pelsy and Caboche, 1992). Mutants lacking NR activity are resistant to chlorate, presumably because they cannot reduce the nitrate analogue to the toxic compound chlorite (Åberg, 1947). Studies by Siddiqi *et al* (1992) showed that chlorate uses the same transporter as nitrate and serves as a substrate for nitrate reductase but does not induce the activity of either and Labrie *et al* (1991) found that chlorate induces nitrate reductase transcript but not nitrate reductase protein or activity. Thus, chlorate is a poor analogue of nitrate and perhaps as a consequence putative nitrate uptake mutants have only been isolated for the low-affinity, inducible, nitrate uptake system (reviewed in Hoff *et al*, 1994). The second method for selection is on the basis of nitrate auxotrophy, where mutants are identified by their inability to grow on a medium containing nitrate as the sole nitrogen source prior to rescue onto ammonium-containing media, and

several nitrate reductase-deficient mutants have been isolated by this method (Strauss *et al*, 1981; Pelsy and Gonneau, 1991). The third method involves selection by assaying nitrate assimilation enzymes for absent or low activity, and is extremely labour intensive, although nitrate reductase-deficient mutants from eight complementation groups have been isolated in this way (Warner and Kleinhoffs, 1992).

#### 1.5.1 Nitrate uptake mutants

Nitrate transport mutants (*nrt* mutants) have been isolated in *Arabidopsis* by resistance to chlorate (Oostinder-Braaksma and Feenstra, 1973; Tsay *et al*, 1993b) and fall into one complementation group. The locus responsible for this chlorate resistance has been designated *Chl1*. The *chl1* mutants are easy to differentiate from other chlorate resistant mutants because they show no chlorosis of the leaves after chlorate treatment. The *Chl1* locus is probably not the only nitrate transport locus in *Arabidopsis* because *chl1* mutants still take up and assimilate nitrate, and grow normally on nitrate suggesting only that *Chl1* is responsible for the major chlorate transport under the experimental conditions used. Kinetic studies of nitrate studies in the *chl1* mutant indicate that it is affected in a low-affinity nitrate uptake system (Doddema and Telkamp, 1979).

#### 1.5.2 Nitrate reductase mutants

The most extensive studies on nitrate reductase mutants in higher plants have been carried out in *Nicotiana*, barley and *Arabidopsis*. Nitrate reductase-deficient mutants isolated to date are of two types; mutations affecting the structural (apoprotein) gene(s) and mutations affecting one of the several genes involved in the biosynthesis of the molybdenum cofactor.

Mutants have been biochemically allocated to one of the two groups depending upon the activity of another MoCo-containing enzyme, xanthine dehydrogenase (XDH). Mutants with a loss of both nitrate reductase and XDH activity are defective in the biosynthesis or processing of the molybdenum cofactor (*cnx* mutants), whereas mutants with a loss of nitrate reductase activity but which possess XDH activity are classified as apoenzyme-deficient mutants (*niam* mutants). The biochemical characterisation has been confirmed with genetic characterisation into separate complementation groups.

#### 1.5.2.1 Apoenzyme-deficient mutants

A great deal of information on nitrate reductase structure and function has been obtained by the study of *nia* mutants. By complementation analysis and biochemical characterisation of *nia* mutants, the number of nitrate reductase apoprotein genes has been determined in a number of species (reviewed in Hoff *et al*, 1994) such as barley, where two *Nia* loci (*nar1* and *nar7*) have been identified and correspond to NADH:nitrate reductase and NAD(P)H:nitrate reductase (Kleinhoffs *et al*, 1989) and both genes have subsequently been cloned (Miyazaki *et al*, 1991; Schnorr *et al*, 1991). The *nar1* mutants were first isolated and shown to lack the NADH:nitrate reductase activity associated with roots and leaves of wild-type plants. Mutagenesis of *nar1* mutant seed yielded a single mutant line, which was also defective in the *Nar7* locus associated with the NAD(P)H:nitrate reductase activity found in the roots.

Intragenic complementation at the *Nia* locus has been demonstrated in *Nicotiana* (Muller and Mendel, 1989; Pelsy and Gonneau, 1991), where mutants affected in different nitrate reductase domains can complement each other *in vivo* by crosses or *in vitro* by mixing protein extracts from the two

mutants. This indicates that electrons are able to flow from one subunit to another and may be the result of either the formation of heterodimers or the interaction between nitrate reductase molecules.

Several *nia* mutant alleles from *Nicotiana* and *Arabidopsis* have been sequenced (Meyer *et al*, 1991; Vaucheret *et al*, 1992b; Meyer *et al*, 1993; Wilkinson and Crawford, 1993) and have allowed greater understanding of the function of some amino acid residues in the nitrate reductase apoprotein (reviewed in Hoff *et al*, 1994).

#### 1.5.2.2 MoCo-deficient mutants

Complementation of MoCo-deficient mutants has identified six *cnx* loci in barley and *Nicotiana* (Dirk *et al*, 1985; Gabard *et al*, 1988; Kleinhoffs and Warner, 1990) and the function of one of these MoCo genes, *Cnx1* in *Arabidopsis thaliana*, has recently been characterised and is probably involved in the insertion of molybdenum into molybdopterin (Stallmeyer *et al*, 1995).

#### 1.5.2.3 Regulation of *nia* mutants

Studies of many *N. plumbaginifolia nia* mutants revealed that nitrate enhances the level of nitrate reductase mRNA in all cases (Pouteau *et al*, 1989). The fact that a whole range of *nia* mutants affected in different domains of the nitrate reductase protein have conserved nitrate-inducibility argues against a direct role of nitrate reductase in its own regulation. This aspect differs from that of *Aspergillus* and *Neurospora*, where nitrate reductase is proposed to autoregulate its own expression (Cove and Pateman, 1969) and many nitrate reductase-deficient mutants express nitrate reductase mRNA constitutively (Hawker *et al*, 1992; Okamoto *et al*, 1993). However, studies have shown that many higher plant *nia* and *cnx* mutants overexpress



nitrate reductase mRNA (Kleinhoffs *et al*, 1989; Pouteau *et al*, 1989; Wilkinson and Crawford, 1991; Labrie *et al*, 1991) and nitrite reductase mRNA (Faure *et al*, 1991; Kronenberger *et al*, 1993). These results provide support for the view that the nitrate assimilation pathway may be down-regulated by an end-product of nitrogen assimilation.

### 1.5.3 Nitrite reductase mutants

Higher plant nitrite reductase-deficient mutants (*nii* mutants) have been more difficult to obtain than *nia* mutants, and only one has been described to date. This is probably because there is no easy selection as for nitrate reductase mutants (chlorate resistance cannot be used) and inactivation of nitrite reductase is conditional-lethal, presumably due to the accumulation of toxic nitrite. Recently, the isolation of a barley mutant (*nir1*) affected in nitrite reduction has been described (Duncanson *et al*, 1993) and will be described in greater detail in Chapter 4. The *nir1* mutant has greatly reduced nitrite reductase activity in both leaf and root, and was isolated from sodium azide-mutagenised seed. Selection of nitrite reductase-deficient mutants was on the basis that lack of nitrite reductase activity leads to accumulation of nitrite in the leaf tissue.

### 1.5.4 Regulatory mutants

No regulatory mutant of the nitrate assimilatory pathway has been identified in higher plants to date. The fact that all of the selections and screenings for regulatory mutants have so far failed (reviewed in Hoff *et al*, 1994) may be because nitrate operates by derepressing the nitrate assimilation pathway (negative control) rather than activating the pathway genes (positive control). If this is the case then screening by chlorate

resistance will not work as defective plants will possess higher rather than lower levels of the affected nitrate assimilation enzymes and hence will display a wild-type phenotype.



## 1.6 STUDY OF NITRATE ASSIMILATION USING TRANSGENIC APPROACHES

The recent cloning of nitrate reductase and nitrite reductase apoprotein genes and cDNA's, along with development of reliable plant transformation systems, has led to the proliferation of studies using transgenic plants. Most of these studies have been performed in tobacco using sense, antisense and promoter deletion strategies.

### 1.6.1 Regulation of nitrate assimilation by nitrate

Transgenic plants have helped to confirm that the regulation of nitrate assimilation enzymes by nitrate is transcriptional. For example, the *N. plumbaginifolia* E23 *nia* (nitrate reductase-deficient) mutant was transformed by a construct where the tobacco *Nia2* cDNA was placed under the control of the CaMV 35S promoter (Vincentz and Caboche, 1991). Analysis of the resultant transgenic plants revealed that *nia* mRNA is expressed constitutively i.e. in the absence of nitrate. This indicates that nitrate effects control of *Nia* genes at the transcriptional level. Furthermore, fusions were made between the tobacco *Nia1* promoter and the GUS reporter gene and introduced into wild-type plants and *nia* (nitrate reductase-deficient) mutants of *N. tabacum* (Vaucheret *et al*, 1992b). Where expression of the transgene was detectable, GUS mRNA could be induced by nitrate in two thirds of the plants. These findings also suggest that nitrate regulation of nitrate reductase is transcriptional.

In the case of nitrite reductase, transgenic tobacco plants were made by integrating a construct where the GUS gene was fused to the spinach *Nii* promoter (Back *et al*, 1991). In this case, the reporter gene expression was

shown to be induced by nitrate so for nitrite reductase also, regulation by nitrate operates, at least partly, at the transcriptional level.

### 1.6.2 Regulation of nitrate assimilation by light

As described previously, light plays a significant role in the regulation of nitrate assimilation genes. Transgenic plants, in which the tobacco *Nia2* cDNA is under control of the CaMV 35S promotor, express high levels of *nia* mRNA in darkness, indicating that light control of *Nia* gene expression is part transcriptional (Vincentz and Caboche, 1991). As discussed previously, sucrose can reproduce the light induction of nitrate reductase activity and mRNA (Cheng *et al*, 1992). Analysis of transgenic tobacco plants containing a fusion of the tobacco *Nia1* promotor and the GUS reporter gene (Vincentz *et al*, 1993), and transgenic *Arabidopsis* plants containing the CAT reporter gene fused to the *Arabidopsis Nia1* promotor (Cheng *et al*, 1992), demonstrated that sucrose control of nitrate reductase expression is, like light control, primarily at the transcriptional level.

Light regulation of nitrate reductase is not purely transcriptional. Transfer to darkness of transgenic plants that constitutively express *nia* mRNA under control of the CaMV 35S promotor results in a decrease of nitrate reductase activity and protein levels but not transcript (Vincentz and Caboche, 1991). This suggests a translational or post-transcriptional regulation of nitrate reductase by light.

### 1.6.3 Regulation of nitrate assimilation by N-metabolites

As described previously, many *nia* and *cnx* mutants overexpress *nia* and *nii* mRNA which indicates a negative feedback mechanism operating on the nitrate assimilation pathway. The same holds true in transgenic tobacco

plants impaired in nitrite reductase activity by the expression of a tobacco leaf *Nii* antisense construct (Vaucheret *et al*, 1992a), where there is overexpression of nitrate reductase activity and mRNA. In the *nia* mutants and the *Nii* antisense transgenic plants, glutamine levels are greatly reduced, confirming glutamine as a possible repressor (Vaucheret *et al*, 1992a; Foyer *et al*, 1993).

#### 1.6.4 *In vivo* promoter analysis

Fusions between full-length or partial *Nia* or *Nii* promoters and a reporter gene have allowed the identification of promoter regions involved in light or nitrate regulation. For example, a 1.4kb region of the tobacco *Nia1* or *Nia2* promoter is sufficient to confer nitrate, glutamine, sugar or circadian regulation to the reporter gene (Vaucheret *et al*, 1992b; Vincentz *et al*, 1993). Deletion analysis of the *Arabidopsis* *Nia1* and *Nia2* gene promoters fused to a reporter gene show that the -238 and -188 bp upstream regions respectively contain sequences responsible for nitrate induction (Lin *et al*, 1994).

For *Nii* genes, a 3.1kb region of the spinach *Nii* promoter is sufficient to confer nitrate inducibility to the GUS reporter gene (Back *et al*, 1991). Moreover, the reporter gene expression is regulated according to *Nii* gene regulation in the tobacco host. That is, GUS expression is enhanced both by nitrate and light in tobacco in contrast to the situation in spinach where *Nii* gene transcription is solely nitrate-dependant (Neininger *et al*, 1993). Promoter deletion analysis of the *Nii* promoter has been performed and 330bp of the spinach *Nii* promoter was shown to be sufficient for nitrate and phytochrome inducibility of GUS expression in transgenic tobacco (Rastogi *et al*, 1993; Neininger *et al*, 1994). So far, promoter sequence comparison between different *Nia* and *Nii* promoters has failed to uncover any significant

homology, apart from a region conserved between the tobacco, tomato and petunia *Nia* promoters (Salanoubat and Dang Ha, 1993).

#### 1.6.5 Study of trans-acting factors

Studies have been performed to postulate a role for a NIT2 protein-like factor in higher plants (Jarai *et al*, 1992). The NIT2 protein of *Neurospora crassa* is involved in the regulation of nitrate reductase by glutamine and binds specifically to the *N. crassa* nitrate reductase promoter region (Fu and Marzluf, 1990). Gel retardation experiments demonstrated that NIT2 binds specifically to two tomato *Nia* promoter fragments (Jarai *et al*, 1992) and these fragments contained motifs identical or similar to the *N. crassa* promoter sequence which binds the NIT2 protein (Fu and Marzluf, 1990). The NIT2 binding site in the *N. crassa* promoter is related to the *I* box (GATAA) which is an important regulatory sequence found in plant light- and circadian clock-responsive promoters (Giuliano *et al*, 1988; Manzara and Gruissem, 1988; Donald and Cashmore, 1990). Further gel retardation experiments performed with tomato nuclear extracts have shown that the *I* box present in the tomato *Nia* promoter is specifically recognised by an uncharacterised protein whose abundance fluctuates following a circadian rhythm (Borello *et al*, 1993), hence this *Nia I* box could define an important region of the tomato *Nia* promoter involved in circadian rhythm regulation. As the *I* box is also present in one of the tomato *Nia* promoter fragments retarded by NIT2 it may also be involved in glutamine regulation of *Nia* expression, which indicates a close interaction of glutamine and circadian regulation for *Nia* gene expression.

## 1.7 AIMS

The aims of the work described here were:

- i. To further characterise the biochemistry and regulation of the whole-plant barley *nir1* mutant STA3999 (Duncanson *et al*, 1993), which is defective in nitrite reduction.
- ii. To identify the genetic location of the *nir1* mutation.
- iii. To biochemically and genetically characterise three further whole-plant barley mutants which accumulate nitrite in the leaf after treatment with nitrate in the light, and to identify the genetic location(s) of any mutation(s).
- iv. To develop a fuller understanding of the molecular biology and regulation of nitrite reduction in wild-type barley plants, which may be used in the characterisation of mutants for this step in nitrate assimilation.

## **CHAPTER 2**

### **Materials and Methods**

## **MATERIALS**

### **2.1.1 Chemicals**

All biochemicals and common chemicals used were of analytical laboratory grade unless otherwise stated.

### **2.1.2 Seed and Plant Growth Materials**

Barley seed of the cultivar Golden Promise and of the cultivars Tweed and Klaxon were obtained from William Watt, Seed Merchants, Cupar, Fife, UK and the Scottish Crop Research Institute, Invergowrie, UK, courtesy of Dr W.T.B.Thomas, respectively.

Vermiculite and Levington M2 potting compost were obtained from the local botanic gardens.

## METHODS

### 2.2 PLANT GROWTH AND MUTANT IDENTIFICATION

#### 2.2.1 Treatment of vermiculite

In order to remove trace amounts of nitrate which may be present, vermiculite was wrapped in muslin then placed in a plastic bin and left to soak in distilled water for 24 hours. This washing step was repeated twice before the vermiculite was removed and left to air dry in a plastic tray. Seed trays were also rinsed thoroughly with distilled water to remove any residual nitrate.

#### 2.2.2 Growth of barley seedlings

Wild-type barley seed of the cultivars Golden Promise, Tweed and Klaxon were sown in clean trays containing washed vermiculite and given a light covering of vermiculite. The seeds were then treated with nitrate-less half-strength Hoaglands nutrient solution (200 $\mu$ M NaFeEDTA, 500 $\mu$ M potassium dihydrogen phosphate, 1mM magnesium sulphate, 400nM zinc sulphate, 200nM manganese sulphate, 20 $\mu$ M boric acid, 50nM sodium molybdate (Hoagland and Arnon, 1938)) in the dark. After 4 days the germinated seedlings were transferred into the light (110 $\mu$ Em<sup>-2</sup>s<sup>-1</sup>) for a further 2 days growth before use. Plants within segregating mutant populations were treated as above, except seeds were sown in unique positions in the tray using a numbered grid (Figure 2.1a).



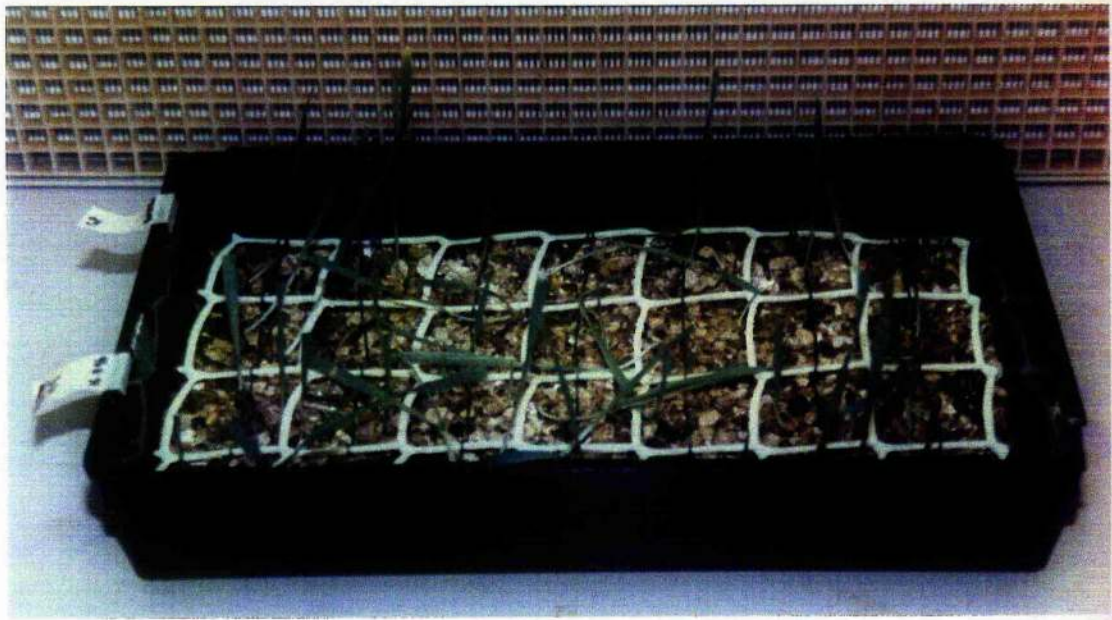
**Figure 2.1 Identification and maintainance of nitrite-accumulating barley mutants**

(a) Seed tray containing 56 plants within a barley population segregating for the leaf nitrite accumulation phenotype, sown in unique positions in a numbered grid.

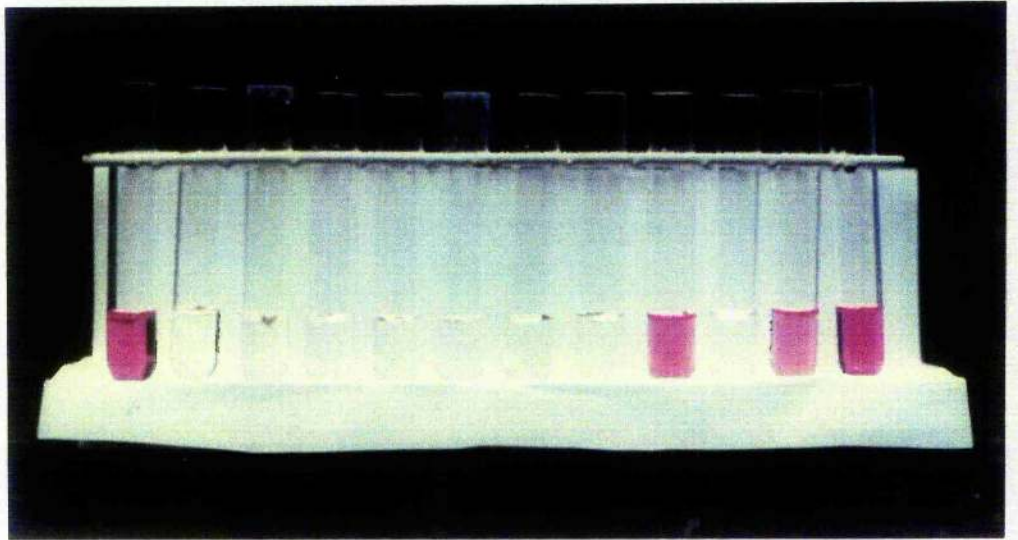
(b) Example of a leaf nitrite accumulation screen. A pink colouration indicates the presence of nitrite in the incubation medium.

(c) Example of a nitrite-accumulating selection maintained in hydroponic culture containing 2mM ammonium chloride as the sole nitrogen source.

(a)



(b)



(c)



### 2.2.3 Screening of mutant populations

Barley mutant populations, segregating for *in vitro* nitrite accumulation, were available for study (Duncanson *et al.*, 1993). Screening for the nitrite accumulation phenotype within these populations was performed according to the method of Duncanson *et al.* (1993). Leaf tips (about 10mm in length) from each plant were placed in separate test tubes to which 1ml of 100mM potassium nitrate was added. The tubes were then placed in the light ( $110\mu\text{Em}^{-2}\text{s}^{-1}$ ) for 16-20 hours. 1ml of 1% (w/v) sulphanilamide in 3N hydrochloric acid and 1ml of 0.02% (w/v) NED were then added to each test tube. Nitrite accumulation was identified by the production of an azo dye with a characteristic pink colour (Snell and Snell, 1949). It was therefore possible to identify the plant or plants displaying the nitrite accumulation phenotype (Figure 2.1b).

## 2.3 GENETIC ANALYSIS

### 2.3.1 Maintenance of nitrite-accumulating selections

In order that back-crosses could be performed between nitrite-accumulating selections and their wild-type cultivars, plants identified as nitrite accumulators were rescued from the vermiculite and grown in the hydroponic system described by Bright *et al* (1983) (Figure 2.1c). Ammonium was used as the sole nitrogen source, due to the poor growth of selections using glutamine as the sole nitrogen source (Duncanson, 1990). The plants were grown in a glasshouse under constant light at a temperature of 20°C with constant aeration of the hydroponic medium (1.3mM potassium dihydrogen phosphate, 100µM NaFeEDTA, 1.5mM magnesium sulphate, 1.2mM calcium chloride, 100µM boric acid, 500nM ammonium molybdate, 18µM manganese sulphate, 2µM zinc sulphate, 2.4µM copper sulphate, 90µM potassium chloride, 5mM MES (pH 6.0), 2mM ammonium chloride). Calcium carbonate (2g) was added to each pot to act as a buffer against the acidic hydrogen ions that are excreted from the roots of ammonium-fed plants. Calcium carbonate is insoluble and will not affect the ionic balance of the hydroponic medium.

To avoid the accumulation of microbial contaminants the solution was changed twice weekly and the plant roots thoroughly rinsed with distilled water. Before reuse, pots were rigorously washed with concentrated hydrochloric acid and thoroughly rinsed with distilled water.

### **2.3.2 Maintenance of wild-type and nitrite non-accumulating selections**

Wild-type and nitrite non-accumulating plants were rescued from vermiculite and planted individually into pots containing potting compost, and grown in a glasshouse under constant light at approximately 20°C.

### **2.3.3 Cross-pollination**

Back-crosses (mutant x wild-type) and allelism test crosses (nitrite non-accumulator x nitrite non-accumulator) were carried out by Dr W.T.B. Thomas at SCRI, Invergowrie, UK.



## 2.4 BIOCHEMICAL ANALYSIS

### 2.4.1 Tissue extraction

Plant tissue (leaf or root) was extracted with either 250mM Tris (pH 8.5), 3mM DTT, 5 $\mu$ M FAD, 1 $\mu$ M sodium molybdate and 1mM Na<sub>2</sub>EDTA (NR extraction buffer; Kuo *et al*, 1980) or 50mM Tris (pH 7.5), 10mM EDTA, 10mM  $\beta$ -mercaptoethanol and 10% (v/v) glycerol (NiR extraction buffer; Ip *et al*, 1990) or 50mM potassium phosphate buffer (pH 7.5), 1mM cysteine, 100 $\mu$ M Na<sub>2</sub>EDTA, 10 $\mu$ M FAD and 3% (w/v) BSA (NR extraction buffer; Small and Wray, 1980) by grinding in a mortar and pestle at a tissue to buffer ratio of 1:5 (g/ml). The homogenate was centrifuged at 30000g for 20 minutes and the supernatant collected as the tissue extract. All procedures were carried out at 4°C.

### 2.4.2 *In vitro* enzyme assays

#### 2.4.2.1 *In vitro* NADH-nitrate reductase

*In vitro* NADH-nitrate reductase activity was assayed according to the method of Wray and Filner (1970), except that the assay reaction was terminated using 200 $\mu$ l of a 1:1 (v/v) mix of 1M zinc acetate and 0.3mM PMS (Scholl *et al*, 1974). The assay mix contained 50mM potassium phosphate buffer (pH 7.5), 10mM potassium nitrate, 200 $\mu$ M NADH and 100 $\mu$ l of tissue extract in a final volume of 1ml. The assay, initiated by the addition of the tissue extract, was performed at 25°C for 20 minutes and terminated as described above. The assay was developed by the addition of 1ml of 1% (w/v) sulphanilamide in 3N hydrochloric acid followed by 1ml of 0.02% (w/v) NED. Controls were treated as above except the enzyme extract was

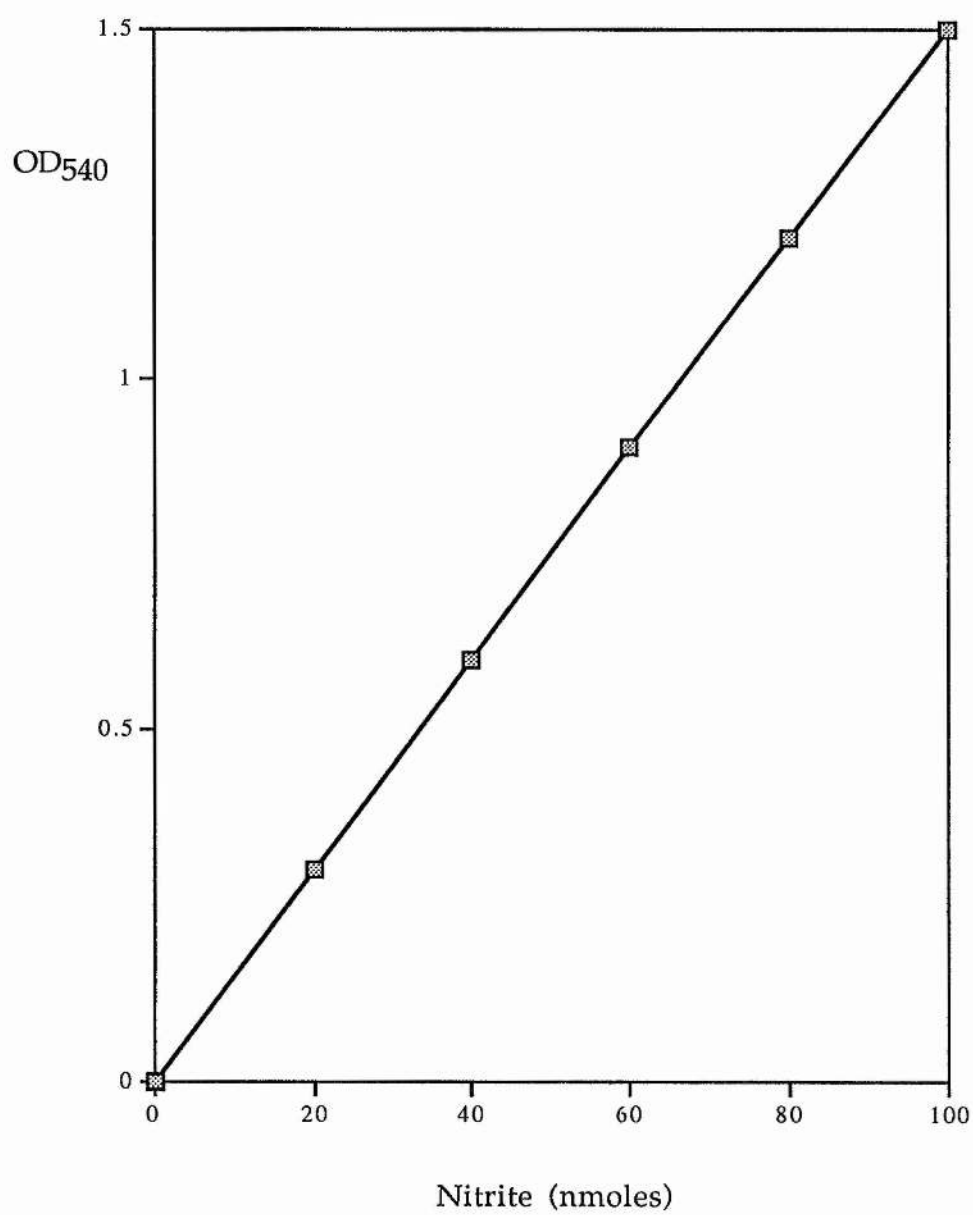


Figure 2.2: Nitrite calibration curve

withheld until after the termination of the assay. Full colour development occurred within 15 minutes, after which the tubes were centrifuged at 1200g in a bench centrifuge for 5 minutes and the OD of the supernatant read at 540nm (Snell and Snell, 1949).

The amount of nitrite produced was calculated using a calibration curve (0-100 nmoles nitrite; Figure 2.2)

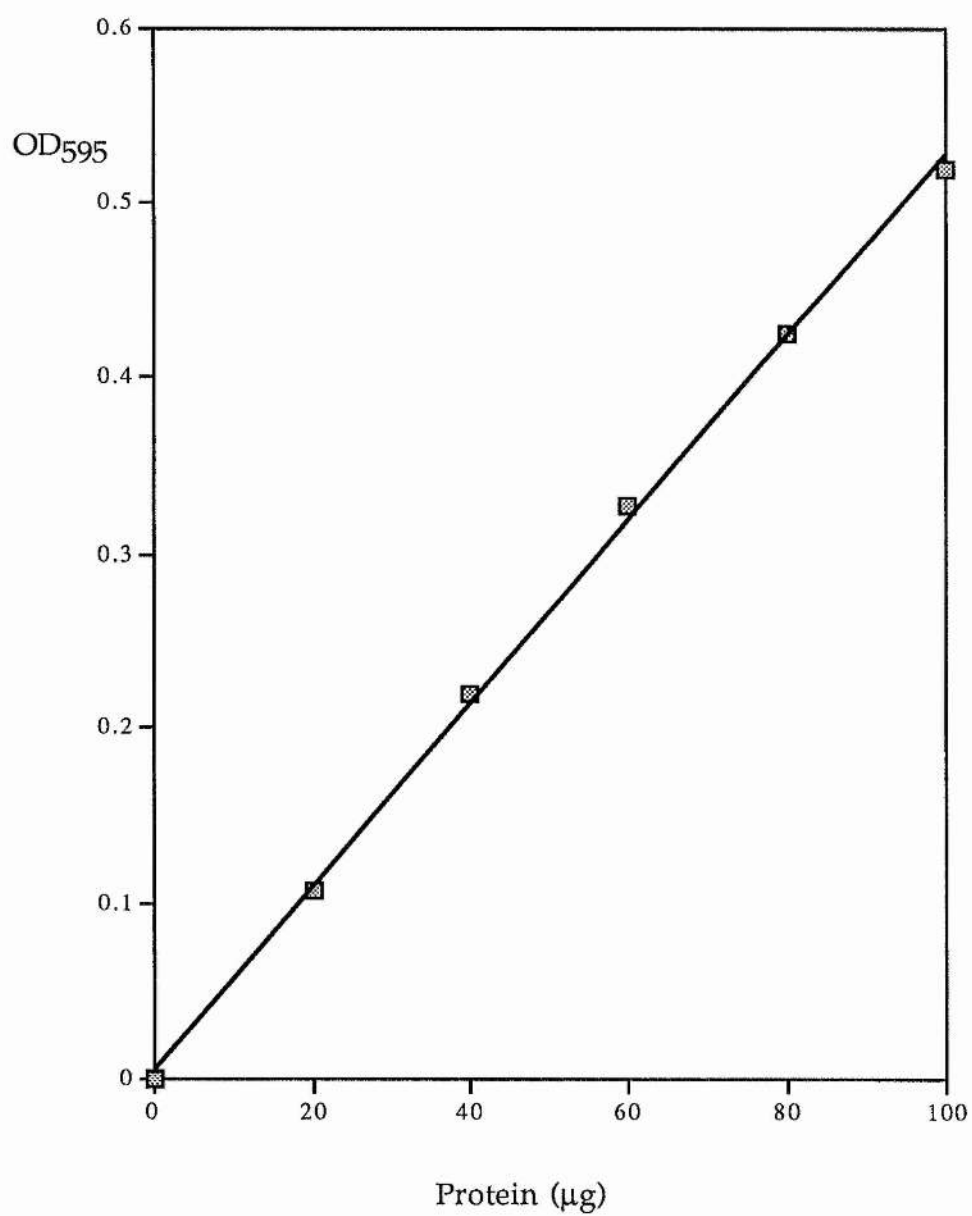
#### 2.4.2.2 *In vitro* NADPH-nitrate reductase

*In vitro* NADPH-nitrate reductase activity was assayed as described above, except that the assay contained 200 $\mu$ M NADPH in place of NADH and also contained 5mM pyruvate and 25 $\mu$ g/ml LDH to remove NADH, formed by the conversion of NADPH to NADH by dephosphorylases in the tissue extract, from the assay (Dailey *et al*, 1982).

#### 2.4.2.3 *In vitro* methyl viologen-nitrite reductase

*In vitro* methyl viologen-nitrite reductase activity was assayed according to the method of Wray and Filner (1970), except the tubes were not flushed with nitrogen. The assay mix contained 50mM potassium phosphate buffer (pH 7.5), 2mM potassium nitrite, 1mM methyl viologen and 100 $\mu$ l of tissue extract in a final volume of 0.8ml. The reaction was initiated by the addition of 200 $\mu$ l of 10mg/ml sodium dithionite in 95mM sodium bicarbonate (test) or 200 $\mu$ l of 95mM sodium bicarbonate (control). The assays were carried out at 25°C for 20 minutes and were terminated by vigorous aeration of the assay mix using a vortex, which oxidised the blue reduced methyl viologen to the colourless leuco form. 30 $\mu$ l of the assay mix was then added to 970 $\mu$ l distilled water followed by 1ml of 1% (w/v) sulphanilamide in 3N hydrochloric acid and 1ml of 0.02% (w/v) NED. Full colour





**Figure 2.3:** Protein calibration curve

development occurred within 15 minutes after which the OD was read at 540nm (Snell and Snell, 1949). The amount of nitrite removed from the assay, hence the amount reduced, was calculated using a calibration curve (0-100 nmoles nitrite; Figure 2.2).

#### **2.4.3 Determination of protein content**

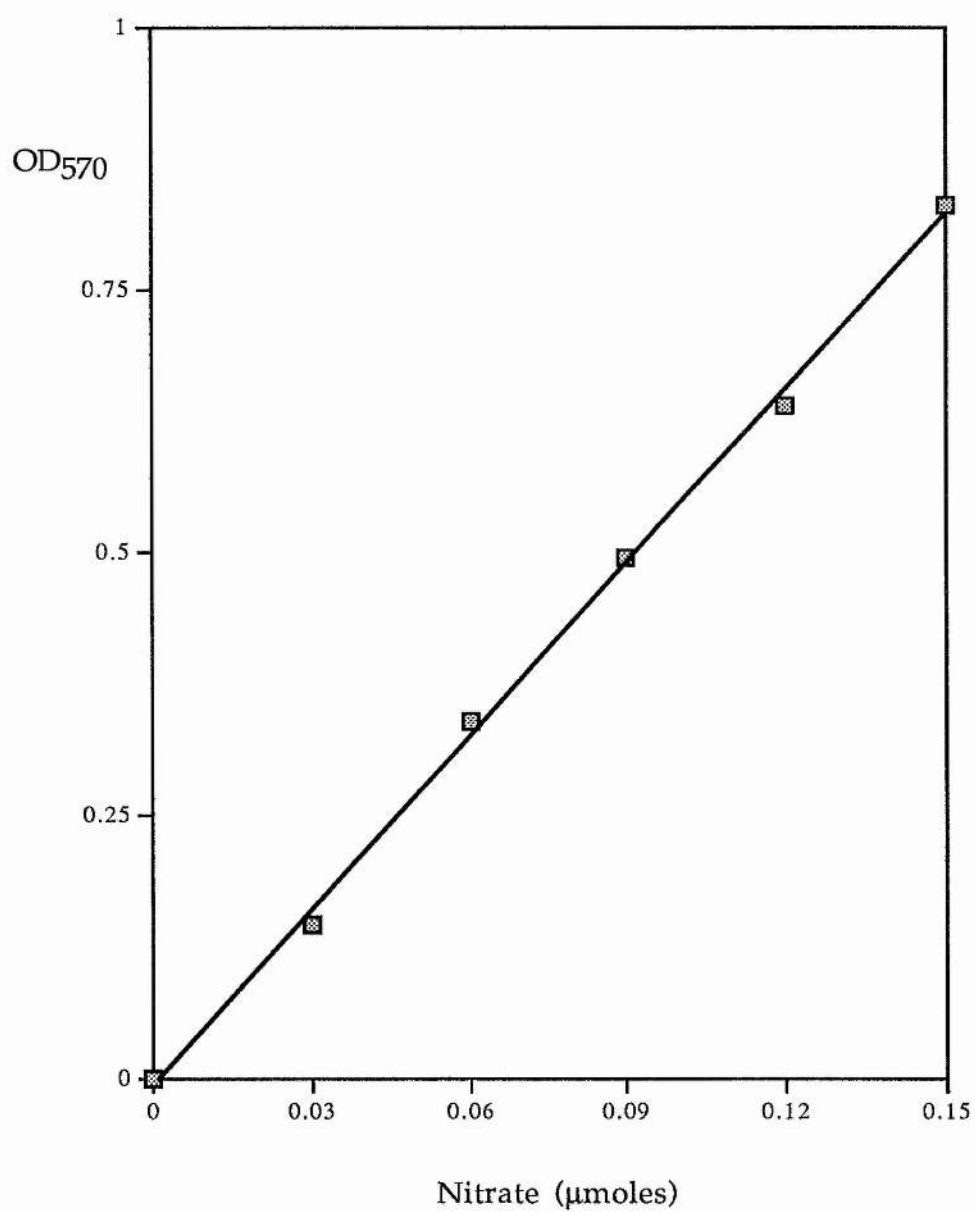
Total protein content of tissue extract was determined using the method of Bradford (1976). The reagent consisted of 100mg of Coomassie brilliant blue G-250, 55ml of ethanol and 110ml of orthophosphoric acid which was made up to a volume of 1 litre with distilled water, and filtered twice through Whatman® No.1 filter paper (Whatman, UK).

Protein content was determined by the addition of 5ml of reagent to 100µl of appropriately diluted sample, followed by mixing. After full colour development, which occurred within 10 minutes, the OD was read at 595nm. Calibration curves (0-100µg protein) were prepared using BSA (Figure 2.3).

#### **2.4.4 Determination of nitrate content**

Nitrate content of tissue extract was determined using the method described by Bright *et al.* (1983). The reagent solution was prepared by mixing equal volumes of concentrated AnalaR™ sulphuric acid and concentrated AnalaR™ orthophosphoric acid in a highly exothermic reaction. Once cool, the reagent solution was stored for at least one week before use.

When ready for use, 0.5% (w/v) diphenylamine sulphonic chromogen (Szechrome NAS®) was added to the reagent solution in a tightly sealed bottle and stirred until the powder dissolved and the liberated gas was absorbed (up to 3 hours), producing a colourless reagent mix. Nitrate



**Figure 2.4:** Nitrate calibration curve

content was determined by the addition of 5ml of the reagent mix to 500 $\mu$ l of appropriately diluted sample, followed by thorough mixing. The OD was read at 570nm within one hour and the nitrate content determined from a calibration curve (0-0.15 $\mu$ moles potassium nitrate; Figure 2.4).

#### 2.4.5 Determination of nitrite content

Pre-weighed leaf tissue (0.1-0.2g) was extracted with 1 ml of distilled water in a microfuge tube and centrifuged at 30000g for 10 minutes. The supernatant was then transferred to a test tube and 1ml of 1% sulfanilamide in 3N HCl and 1ml of 0.02% NED was added and the OD was read at 540nm (Snell and Snell, 1949). Nitrite content was measured using a calibration curve (0-100nmoles nitrite; Figure 2.2).

#### 2.4.6 Immunoblot analysis

##### 2.4.6.1 SDS-polyacrylamide gel electrophoresis

Vertical slab gels were prepared essentially as described by Laemmli (1970). The separating gel, containing 10% (w/v) acrylamide, 0.25% (w/v) methylene-bisacrylamide, 375mM Tris (pH 8.8), 12% (w/v) sucrose and 0.1% (w/v) SDS, was polymerised by the addition, to a final concentration, of 0.1% (w/v) ammonium persulphate and 0.0005% (v/v) TEMED. The stacking gel, containing 4.8% (w/v) acrylamide, 0.13% (w/v) methylene-bisacrylamide, 175mM Tris (pH 6.8), 12% (w/v) sucrose and 0.08% (w/v) SDS, was polymerised by the addition, to a final concentration, of 0.08% (w/v) ammonium persulphate and 0.0005% (v/v) TEMED.

Samples of tissue extract containing 50 $\mu$ g of total protein were prepared for loading by adding a quarter volume of 5x sample loading

buffer (300mM Tris (pH 6.8), 25% (v/v) glycerol, 10% (w/v)  $\beta$ -mercaptoethanol, 10% (w/v) SDS and 0.05% (w/v) bromophenol blue (Davis *et al*, 1986)) in a microfuge tube. These samples, along with a 10 $\mu$ l sample of pre-stained SDS-PAGE standard solution (Sigma, UK), were placed in a boiling water bath for 2 minutes then removed and allowed to cool for 5 minutes before loading onto the gel.

Electrophoresis was performed at 10mA for 5 hours and 2mA overnight in a running buffer containing 25mM Tris (pH 8.3), 192mM glycine and 0.1% (w/v) SDS.

#### 2.4.6.2 Protein electrotransfer (western blotting)

Proteins were transferred from the acrylamide gels onto nitrocellulose filters essentially as described by Towbin *et al* (1979). The acrylamide separating gel was removed from the SDS-PAGE apparatus and placed against two pre-wetted sheets of Whatman<sup>®</sup> 3MM filter paper cut to fit the electrotransfer cassette, taking care to remove any air bubbles. A nitrocellulose filter (Schleicher & Schuell, Germany) was cut to a slightly larger size than the gel, pre-wetted and placed against the gel. This was followed by two further pieces of pre-wetted Whatmann 3MM filter paper cut to fit the cassette. The gel sandwich was then placed between two Scotchbrite pads and inserted into the cassette, then placed in the electrotransfer tank.

Transfer was performed at a constant current of 200mA for 3 hours in a transfer buffer containing 20mM Tris (pH 8.3), 193mM glycine and 20% (v/v) methanol.

#### 2.4.6.3 Development of electroblots for nitrite reductase cross-reacting material

All incubations and washes of nitrocellulose filters were performed in shallow trays placed on a low speed shaker table at room temperature. The nitrocellulose filters were first incubated in trays containing excess blocking buffer (TBST (100mM Tris buffer (pH 8.0), 15mM sodium chloride, 0.05% (v/v) Tween-20) containing 4% (w/v) Marvel) for 60 minutes. This solution was replaced with 100ml of TBST containing 1% (w/v) Marvel and the polyclonal anti-barley nitrite reductase antiserum (1:50000 dilution; Duncanson *et al*, 1992), raised in rabbit against purified barley nitrite reductase enzyme (Ip *et al*, 1990), and incubated overnight. The filters were then washed with three changes (20 minutes each) of excess (50-100ml) TBST before incubating with a 1:2000 dilution of alkaline phosphatase-linked anti-rabbit IgG (Sigma, UK) in 100ml TBST containing 1% (w/v) Marvel for 2 hours. The filters were again washed with three changes (20 minutes each) of excess (50-100ml) TBST. The nitrocellulose filters were then developed with NBT (33mg%) and BCIP (disodium salt, 10.5mg%) in 80ml AP buffer (100mM Tris (pH 9.5), 100mM sodium chloride, 5mM magnesium chloride). The alkaline phosphatase enzyme converts the BCIP to a blue indigo dye which releases hydrogen ions that reduce the NBT salt to the corresponding, intensely purple, diformazan. Both these compounds are deposited at the site of alkaline phosphatase activity, producing purple bands at the protein-antibody recognition site. The reaction was stopped by thoroughly washing the filter with excess distilled water.

## 2.5 NUCLEIC ACID ANALYSIS

### 2.5.1 Total RNA extraction

Two RNA extraction methods were used for northern analysis. The miniprep method (2.5.1.1) gave lower RNA returns (approximately 40µg) than the Guanidine-HCl (2.5.2.2) method (approximately 100µg) but the amount of tissue required was lower which made the miniprep method more suitable for some of the mutant characterisation work where the amount of mutant tissue available was limited.

#### 2.5.1.1 Total RNA extraction (*miniprep method*)

Total RNA was extracted from leaf tissue according to the method of Voerwerd *et al* (1989). Leaf tissue (100mg) was frozen in liquid nitrogen and homogenised with 500µl of pre-warmed (80°C) extraction buffer (50% (v/v) phenol (pH>7.8), 50mM lithium chloride, 50mM Tris (pH 8.0), 5mM Na<sub>2</sub>EDTA, 0.05% (w/v) SDS) in a microfuge tube. The homogenate was mixed with 250µl of chloroform and centrifuged at 10000g for 5 minutes at room temperature. The upper phase was then transferred to a fresh tube and the total RNA was precipitated by the addition of an equal volume of 4M lithium chloride and incubated overnight at 4°C.

RNA was then pelleted at 10000g for 10 minutes at 4°C. The pellet was vacuum-dried and resuspended in 0.25ml of DEPC-treated sterile distilled water. RNA was then precipitated by the addition of 0.1 volumes of 3M sodium acetate (pH 5.2) and 2 volumes of ethanol followed by incubation for 2 hours at -20°C. The sample was then centrifuged at 10000g for 10 minutes at 4°C and the pellet was washed with 70% (v/v) ethanol, air-dried

and resuspended in 50 $\mu$ l of DEPC-treated sterile distilled water (see Appendix).

#### 2.5.1.2 Total RNA extraction (Guanidine-HCl method)

Total RNA was extracted from leaf tissue using the Guanidine-HCl method essentially as described by Logemann *et al* (1979), except that RNA precipitation steps were carried out in 2M lithium chloride rather than in ethanol. Leaf tissue (500mg), frozen in liquid nitrogen, was homogenised with 400 $\mu$ l of extraction buffer (8M guanidine-HCl (pH 7.0), 50mM  $\beta$ -mercaptoethanol, 20mM MES, 20mM Na<sub>2</sub>EDTA) and incubated for 10 minutes at room temperature in a microfuge tube. The homogenate was mixed with 700 $\mu$ l of phenol/chloroform (1:1, v/v) and incubated for 10 minutes at room temperature before being centrifuged at 10000g for 10 minutes at room temperature. The upper phase was transferred to a sterile microfuge tube and the RNA was precipitated by the addition of 0.33 volumes of 8M lithium chloride and incubated at 4°C overnight.

RNA was then pelleted at 10000g for 10 minutes at 4°C and the pellet washed with 2M lithium chloride, vacuum-dried and resuspended in 400 $\mu$ l of resuspension buffer (40mM Tris (pH 7.5), 20mM sodium acetate, 5mM Na<sub>2</sub>EDTA, 1% (w/v) SDS). RNA was then precipitated by the addition of 0.1 volumes of 3M sodium acetate (pH 6.0) and 2 volumes of ethanol then incubated at -20°C for 2 hours. The RNA was pelleted at 10000g for 10 minutes at 4°C and the pellet was washed in 70% (v/v) ethanol, air-dried, then resuspended in 50 $\mu$ l DEPC-treated sterile distilled water (see Appendix).



### 2.5.2 Genomic DNA extraction

Genomic DNA was extracted from leaf tissue using the CTAB extraction method of Dean *et al.* (1992). Leaf tissue (1g), frozen in liquid nitrogen, was homogenised with 6.25ml of CTAB buffer (220mM Tris (pH 8.0), 0.8% (w/v) CTAB, 800mM sodium chloride, 140mM sorbitol, 1% (w/v) sarkosyl, 22mM Na<sub>2</sub>EDTA) in a liquid nitrogen-cooled Corex<sup>®</sup> tube and incubated at 65°C with regular shaking for 20 minutes. Following the addition of 2.5ml of chloroform the homogenate was incubated at room temperature with constant shaking for 20 minutes before centrifugation at 5000g for 5 minutes at room temperature. The aqueous phase was transferred to a fresh Corex<sup>®</sup> tube containing an equal volume of isopropanol, incubated on ice for 10 minutes and then centrifuged at 5000g for 5 minutes at 4°C. The resultant pellet was dried before resuspension in 1ml TE Buffer (See Appendix). RNA was then precipitated by the addition of an equal volume of 4M lithium chloride and the sample incubated on ice for a further 20 minutes before centrifugation at 10000g for 10 minutes at 4°C. The supernatant was transferred to a fresh Corex tube and the DNA precipitated by the addition of 0.1 volumes of 3M sodium acetate (pH 6.0) and 2 volumes of ethanol, then incubated at -20°C overnight.

The sample was then centrifuged at 10000g for 10 minutes at 4°C and the DNA pellet washed with 70% (v/v) ethanol before air-drying and resuspending in 250µl of TE buffer (see Appendix).

## 2.5.3 Determination of nucleic acid concentration and quality

### 2.5.3.1 Spectrophotometric determination

The nucleic acid content of samples was assessed by measuring the OD at 260nm, essentially as described by Sambrook *et al* (1989). The conversion factor for RNA at 260nm is 40µg/ml per OD unit and 50µg/ml per OD unit for DNA. For example, if 10µl of RNA is added to 990µl of water and the OD read at 260nm, then:

$$\begin{aligned}\text{RNA yield (}\mu\text{g/ml)} &= \text{OD at 260nm} \times \text{dilution factor} \times \text{conversion factor} \\ &= \text{OD at 260nm} \times (1000/10) \times 40 \\ &= \text{OD at 260nm} \times 400\end{aligned}$$

The quality of the sample is determined by the ratio of the optical density at 260nm to that at 280nm. The closer the ratio is to 2 the greater the purity of the sample. After spectrophotometric analysis, RNA samples were resuspended at a concentration of 1µg/µl by the addition of the appropriate volume of DEPC-treated sterile distilled water.

### 2.5.3.2 Minigel analysis

The quality of nucleic acid samples were checked visually using agarose gel electrophoresis. A 1µl amount of the nucleic acid sample, containing 1µg nucleic acid, was added to 8µl of sterile distilled water and 1µl of 10x electrophoresis loading buffer (see Appendix). A sample containing 0.5µg of *Hind*III-digested λ DNA (Gibco-BRL, UK) was prepared in the same way and used as a standard. The standard and test samples were fractionated through a 0.8% (w/v) agarose/1x TAE minigel (see Appendix),

using 1x TAE (see Appendix) as running buffer, at 80mA until the dye front was 2/3 to 3/4 of the gel length. The gel was removed and stained in a 1µg/ml solution of ethidium bromide for 10 minutes, followed by destaining in water for 20 minutes. The gel was then visualised on a UV transilluminator and photographed. Both the quality (lack of degradation) and quantity (as compared to the standard) could then be assessed.

## 2.5.4 Northern blotting

### 2.5.4.1 Agarose gel electrophoresis of total RNA

A 10µg amount of each RNA sample was added to an equal volume of 2x RNA sample buffer (50% (v/v) deionised formamide, 16.5% (v/v) formaldehyde, 10mM Na<sub>2</sub>EDTA (pH 7.0), 40mM sodium dihydrogen phosphate (pH 6.8), 0.02% (w/v) ethidium bromide) was added. Also, 3µg of RNA ladder markers (Gibco-BRL, UK) were mixed with an equal volume of 2x RNA sample buffer and all samples were incubated at 65°C for 15 minutes. After this period, samples were immediately placed on ice and allowed to cool for 5 minutes before the addition of 4µl of RNA loading dye (0.25% (v/v) bromophenol blue, 8% (w/v) sucrose).

The RNA samples were then loaded onto a 1% (w/v) agarose/1x MOPS gel containing 7% formaldehyde. The gel was run at 70V for 3 hours with recirculation of the 1x MOPS (20mM MOPS (pH 7.0), 5mM sodium acetate, 1mM Na<sub>2</sub>EDTA) running buffer. After this time the gel was removed, and the RNA visualised on a UV transilluminator and photographed, noting the distance of migration of the markers so that the size of any future hybridising band could be estimated. Visualisation also provided a means for checking that equal amounts of RNA had been loaded.

#### 2.5.4.2 Gel blotting

After visualisation the gel was washed in distilled water ( $2 \times 10$  min) to remove the formaldehyde. Transfer of total RNA was carried out using essentially the Hybond<sup>TM</sup>-N protocol (Amersham, UK).

A large plastic tray was filled with 20x SSC (see Appendix) and a perspex platform, covered in a wick consisting of 2 sheets of Whatman<sup>®</sup> 3MM filter paper (Whatman, UK) pre-wetted in 20x SSC, was placed on top. The gel was placed face-down on the wick, taking care to avoid trapping air bubbles beneath. Cling film was then placed around the gel to ensure the transfer buffer was channelled through it. A sheet of Hybond<sup>TM</sup>-N membrane (Amersham, UK), cut to the same size as the gel, was placed on top and any air bubbles were squeezed out using a Pasteur pipette. Two sheets of pre-wetted, then two sheets of dry, 3MM paper cut to the same size were placed on top of the membrane, and a stack of absorbent paper towels, approximately 10cm high placed onto these. Finally, a perspex plate supporting a 0.75-1kg weight was placed onto the stack of towels and the nucleic acid was left to transfer to the membrane for 16-20 hours.

The following day, the apparatus was dismantled and, prior to removing the gel, the orientation of the membrane was marked to allow the identification of the gel lanes after hybridisation. The membrane was removed and air-dried face up for 1 hour before fixing in a Spectrolinker<sup>TM</sup> XL-1500 UV crosslinker (Scotlab, UK).

## 2.5.5 Southern blotting

### 2.5.5.1 Digestion of genomic DNA

A 15µg sample of genomic DNA was made up to 34µl with sterile distilled water and the following added:-

4µl	10x restriction buffer	(Promega, UK)
1µl	RNAse A	(Gibco-BRL, UK)
1µl	restriction enzyme (20U µl <sup>-1</sup> )	(Promega, UK)

The sample was mixed and incubated in a 37°C heating block overnight. The enzyme was inactivated by the addition of Na<sub>2</sub>EDTA (pH 7.5) to a final concentration of 10mM.

The length of time required for complete digestion of the DNA could be estimated as 1U of restriction enzyme digests 1µg DNA/hr (Sambrook *et al*, 1989). The above digestion contains 20U of enzyme and 15µg DNA, hence the digestion should be completed in 45 minutes. However, a digestion excess of at least ten times is normally recommended, so the minimum recommended time for this digestion would be 7 hours and 30 minutes

### 2.5.5.2 Agarose gel electrophoresis of genomic DNA

Gel electrophoresis of genomic DNA is the same as the general method described in section 2.5.3.2, except that 0.1 volumes of 10x electrophoresis loading buffer (see Appendix) was added to each 15µg DNA digest before loading onto a 0.8% (w/v) agarose/1x TAE gel. However, the standard markers were prepared in the same way as previously described (2.5.3.2).

### 2.5.5.3 Gel blotting

Transfer of genomic DNA was carried out using the same general procedure described in section 2.5.4.2, except that after visualisation of the DNA the gel was washed in two changes (2 x 15 minutes) of a denaturing solution (1.5M sodium chloride, 500mM sodium hydroxide) and two changes (2 x 15 minutes) of a neutralising solution (1.5M sodium chloride, 500mM Tris (pH 7.2), 1mM Na<sub>2</sub>EDTA) before setting up the blot as previously described (2.5.4.2).

## 2.5.6 Construction of radiolabelled probes

### 2.5.6.1 Isolation of cDNA inserts

*E. coli* containing the cDNA clone of interest were streaked onto a 1.5% (w/v) LB agar (see Appendix) plate containing the appropriate selective antibiotic (see Appendix) and incubated overnight at 37°C. 5ml of LB broth (see Appendix) containing the same selective antibiotic was inoculated with a single, well isolated colony from the plate and incubated with shaking at 37°C for 12-16 hours. The culture was then centrifuged at 3000g for 5 minutes and the supernatant was discarded.

The plasmid contained within the bacterial cells was isolated using a QIAprep Spin Plasmid™ Miniprep kit (Qiagen, UK) according to the manufacturers instructions. This method involves alkaline lysis of the bacterial cell and isolation of the plasmid in a high-affinity Sephadex resin column which is subsequently eluted under low salt conditions, yielding up to 20µg of plasmid in 50µl of TE buffer (see Appendix).

The plasmid was then digested as follows:-

10µl	plasmid sample (3-4µg)	
7µl	sterile distilled water	
2µl	10x restriction buffer	(Promega, UK)
1µl	restriction enzyme (20U/µl)	(Promega, UK)

The digest was terminated by the addition of Na<sub>2</sub>EDTA to a final concentration of 10mM, and prepared for electrophoresis by adding 3µl of 10x electrophoresis loading buffer (see Appendix). The digest was then fractionated through a 0.8% (w/v) agarose minigel as described in section 2.5.3.2. After visualisation of the DNA, the insert of interest was excised from the gel using a scalpel and placed in a microfuge tube. The DNA was then eluted from the gel slice using a QIAEX II™ Gel Extraction kit (Qiagen, UK) according to the manufacturers instructions. The QIAEX II™ kit uses a high-affinity Sephadex resin to bind the DNA in the presence of agarose, which is later eluted by incubating with a low salt buffer. As the yield of this step depends on the size of the insert relative to the size of the vector, the concentration of the insert was estimated on a minigel, as described in section 2.5.3.2. These isolated cDNA inserts could then be used as double-stranded cDNA probes.

#### 2.5.6.2. *Synthesis of random oligonucleotide-primed probes*

Radiolabelling of double-stranded DNA probes was performed using the Amersham Multiprime DNA Labelling System (Amersham, UK) essentially to the manufacturers instructions. A 25ng amount of the probe was made up to 28µl with sterile distilled water, denatured in a boiling water



bath for 5 minutes and immediately cooled on ice. The following were then added to set up a labelling reaction:-

10µl	10x labelling buffer	
5µl	Primer/BSA	
5µl	$^{32}\text{P}$ dCTP (3000 Ci mMol <sup>-1</sup> )	(ICN, UK)
2µl	Klenow polymerase	

This labelling reaction was incubated at 37°C for 30-45 minutes. Dextran Blue dye (10µl of a saturated solution) was added to the labelling reaction and the reaction volume was passed through a Nick<sup>TM</sup> column (Pharmacia, UK) containing Sephadex<sup>®</sup>-G50. The purified, radiolabelled fragment formed by the reaction passes through the column with the dye and this fraction was collected and stored at -20°C until needed for use in hybridisation.

## 2.5.7. Hybridisation of nucleic acids and autoradiography

### 2.5.7.1. Northern hybridisation

Northern blots were hybridised in a Techne hybridisation oven (Hybaid, UK) using essentially the Hybond<sup>TM</sup>-N protocol (Amersham, UK). The filter was left to pre-hybridise at 42°C in 25ml of pre-hybridisation solution (5x SSPE (see Appendix), 5x Denhardt's (0.1% BSA, 0.1% Ficoll<sup>TM</sup>, 0.1% PVP), 0.5% SDS, 500µg denatured salmon sperm) for 3-4 hours. The appropriate radiolabelled probe was synthesised as described above, denatured in a boiling water bath for 5 minutes and immediately cooled on ice. The denatured probe was then added to the pre-hybridisation solution and allowed to hybridise to the filter overnight.



The filter was then removed from the hybridisation solution and washed with a solution containing 2x SSPE and 0.1% (w/v) SDS (2 x 15 minutes), then with a solution containing 1x SSPE and 0.1% (w/v) SDS (2 x 15 minutes) at 42°C. A further wash with 0.2x SSPE and 0.1% (w/v) SDS was generally not necessary. The filter was then heat-sealed in a plastic sleeve and placed between two sheets of Kodak X-OMAT™ film (Kodak, UK) in an autoradiographic cassette and stored at -80°C. The top sheet of film was developed after 24 hours exposure and used to estimate the optimum exposure time for the bottom sheet of film. Film development was performed using an automated developer.

#### *2.5.7.2. Southern hybridisation*

The hybridisation of Southern blots with homologous probes was performed as described above except that the pre-hybridisation solution did not contain deionised formamide, the filters were washed down to 0.2x SSPE and 0.1% (w/v) SDS and all hybridisation and wash procedures were carried out at 65°C. The hybridisation of Southern blots with heterologous probes was performed as for homologous probes, except that all hybridisation and wash procedures were carried out at 55°C.

#### **2.5.8. Removal of radiolabelled probes from filters**

Northern and Southern filters were stripped according to the Hybond™-N protocol (Amersham, UK). A 250ml volume of 0.1% SDS solution was boiled and poured onto the appropriate filter in a glass dish and left to cool to room temperature. The filter was then heat-sealed in a plastic sleeve and, when high activity probes were being removed, placed

against Kodak X-OMAT™ film in an autoradiographic cassette and exposed overnight at -80°C to ensure there was no remaining radioactivity.

## 2.6 PREPARATION OF A cDNA LIBRARY

### 2.6.1 Large-scale total RNA extraction from leaf tissue

Extraction of total RNA from leaf tissue was performed according to the method of N. Harris, University of St Andrews (personal communication). Leaf tissue (5g), frozen in liquid nitrogen, was ground to a fine powder in a pre-cooled mortar and pestle. The sample was then transferred to a mortar and pestle at room temperature containing 50ml of homogenising buffer (1% (w/v) tri-isopropyl naphthalene sulphonic acid (sodium salt), 6% (w/v) 4-amino salicylic acid, 5% (v/v) phenol (pH>7.8), 50mM Tris (pH 8.5)) and homogenised with the addition of 50ml of phenol/chloroform (1:1,v/v). This suspension was transferred to a fresh Corex™ tube and centrifuged at 7500g for 20 minutes at 4°C and the resulting supernatant was then mixed with an equal volume of phenol/chloroform (1:1, v/v) before centrifugation at 1800g for 10 minutes at room temperature. The aqueous layer was removed and re-extracted with phenol/chloroform (1:1, v/v) followed by extraction with a half-volume of chloroform. The aqueous layer was retained and the nucleic acid precipitated by the addition of 0.1 volumes of 3M sodium acetate (pH 6.0) and 2 volumes of ethanol at -20°C overnight.

The nucleic acid was then pelleted at 12000g for 15 minutes at 4°C and the pellet washed with 70% (v/v) ethanol before resuspension in 1ml of DEPC-treated sterile distilled water. An equal volume of 2x CTAB buffer (2% (w/v) CTAB, 100mM Tris (pH 8.0), 20mM Na<sub>2</sub>EDTA, 1.4M sodium chloride) was added, followed by 2 volumes of CTAB precipitation buffer (1% (w/v) CTAB, 50mM Tris (pH 8.0), 10mM Na<sub>2</sub>EDTA). In order to isolate RNA from the precipitate, the precipitate was pelleted at 12000g for 30 minutes at 4°C and the supernatant discarded. After resuspension of the pellet in 1ml 1x

CTAB buffer (1% (w/v) CTAB, 50mM Tris (pH 8.0), 10mM Na<sub>2</sub>EDTA, 700mM sodium chloride) RNA was reprecipitated by the addition of an equal volume of CTAB precipitation buffer before recentrifugation at 12000g for 15 minutes at 4°C. The pellet was resuspended in 1ml of 1.4M sodium chloride (pH 6.0) before the addition of 2.5 volumes of ethanol to precipitate the RNA. The sample was then incubated at -20°C overnight.

The sample was then pelleted at 12000g for 15 minutes at 4°C and dried under vacuum. The desiccated pellet was resuspended in 500µl of DEPC-treated sterile distilled water (see Appendix) before an equal volume of 8M lithium chloride was added and the mixture incubated at -20°C for 2 hours. This lithium chloride precipitation step was repeated and then the sample was ethanol/sodium acetate precipitated overnight as before. The RNA was pelleted at 12000g for 15 minutes at 4°C, washed in 70% (v/v) ethanol, dried under vacuum then resuspended in 1ml DEPC-treated sterile distilled water. The RNA content was determined as described in section 2.5.3.

## **2.6.2 Large-scale total RNA extraction from root tissue**

Extraction of total RNA from root tissue was performed according to the method of N. Harris, University of St Andrews, UK (personal communication). Root tissue (8g), frozen in liquid nitrogen, was ground to a fine powder using a pre-cooled mortar and pestle, then transferred to a fresh mortar and pestle at room temperature containing 4.8ml NTES buffer (10mM Tris (pH 7.5), 100mM sodium chloride, 1mM EDTA (pH 7.5), 1% (w/v) SDS) and 4.8ml phenol/chloroform (1:1/v:v) and ground to a liquid, transferred to a centrifuge tube and homogenised by vortexing. The sample was then centrifuged at 10000g for 10 minutes at 4°C. The aqueous phase was removed and stored on ice. A further 4.8ml of NTES buffer was added to the

lower phase, vortexed and centrifuged at 10000g for 5 minutes at 4°C. The upper aqueous layer was removed and added to that obtained previously. To this, an equal volume of phenol/chloroform (1:1, v/v) was added and the mixture was vortexed before centrifugation at 3000g for 5 minutes. The upper aqueous layer was transferred to a sterile tube and the nucleic acid precipitated in the by the addition of 0.1 volumes of 3M sodium acetate (pH 6.0) and 2.5 volumes of ethanol, then incubated overnight at -20°C.

The nucleic acid was then pelleted at 3000g for 20 minutes at 4°C, the pellet washed with 70% (v/v) ethanol and dried under vacuum. The pellet was resuspended in 1ml of DEPC-treated sterile distilled water and RNA was precipitated by the addition of an equal volume of 4M lithium chloride and incubation on ice for 3 hours before pelleting at 3000g for 20 minutes at 4°C and drying the RNA pellet under vacuum. The pellet was then resuspended in 1ml of DEPC-treated sterile distilled water and the RNA was reprecipitated and dried as described above. The RNA pellet was then resuspended in 1ml of DEPC-treated sterile distilled water and the RNA precipitated by the addition of 0.1 volumes of 3M sodium acetate (pH 6.0) and 2.5 volumes of ethanol followed by incubation at -20°C overnight.

The sample was then centrifuged at 3000g for 20 minutes at 4°C and the pellet was washed with 70% (v/v) ethanol, dried under vacuum and resuspended in 1ml of DEPC-treated sterile distilled water. The RNA content was determined as described in section 2.5.3.

### 2.6.3 Poly A<sup>+</sup> RNA isolation

Poly A<sup>+</sup> RNA was purified from total RNA using a QIAGEN Oligotex-dT™ kit (Qiagen, UK) according to the manufacturers instructions. This method uses a high-affinity Sephadex resin to hybridise and isolate RNA possessing a poly A<sup>+</sup> tail from total RNA. The poly A<sup>+</sup> RNA was

subsequently eluted by destabilising the dT:rA hybrids with a low-salt elution buffer.

The poly A<sup>+</sup> RNA content of the sample was determined as described in section 2.5.3.1 except that the whole sample was resuspended in 1ml of DEPC-treated sterile distilled water and the OD read at 260nm and 280 nm in a sterile quartz cuvette. The poly A<sup>+</sup> RNA was then precipitated by the addition of 0.1 volumes of 3M sodium acetate (pH 6.0) and 2 volumes of ethanol followed by incubation at -20°C overnight.

The sample was then centrifuged at 10000g for 10 minutes at 4°C and the resulting pellet was washed with 70% (v/v) ethanol and air-dried before resuspending at 1µg/µl in DEPC-treated sterile distilled water. The quality of the poly A<sup>+</sup> RNA was then assessed by using 1µg for minigel analysis as described in section 2.5.3.2.

#### 2.6.4 cDNA library construction

The construction of a Uni-ZAP<sup>TM</sup> XR cDNA library from the poly A<sup>+</sup> RNA was carried out by the custom library service at Stratagene, UK. The Uni-ZAP<sup>TM</sup> XR cloning system is based on the λZAPII vector, from which the pBluescript SK(-) plasmid can be excised. The cDNA was synthesised using a mixture of oligo-dT and random primers, and was size-fractionated to >400bp and cloned unidirectionally in the sense orientation into the *Eco*RI and *Xho*I sites of the λZAPII vector with respect to the *lacZ* promoter. Aliquots of both the unamplified and amplified libraries were provided and were stored as 1ml aliquots after the addition, to a final concentration, of 0.3% (v/v) chloroform (for short term storage at -20°C) or 7% (v/v) DMSO (for long term storage at -80°C).

## 2.7 cDNA LIBRARY SCREENING

All of the following procedures refer to the amplified library only and were performed according to the manufacturers instructions (Stratagene, UK) unless otherwise stated. All bacterial and phage strains used therein were provided by Stratagene, UK.

### 2.7.1 Preparation of plating cultures

Twenty-four hours prior to plating out the library, a 10ml culture of LB broth (see Appendix) supplemented with 0.2% (w/v) maltose and 10mM magnesium sulphate was inoculated with a single colony of XL-1 Blue cells and incubated overnight at 30°C with shaking.

The cells were then pelleted at 3000g for 5 minutes and resuspended in the volume of 10mM magnesium sulphate required to give an OD of 0.5 at 600nm.

### 2.7.2 Library titre

The following dilutions of the amplified library were made: 1:1000, 1:10000 and 1:100000. A 1µl sample of each dilution was placed in a Falcon tube and was incubated with 200µl of OD<sub>600</sub>=0.5 XL-1 Blue host cells for 25 minutes with shaking at 37°C. To each, 3.5ml of pre-heated (50°C) top agarose (see Appendix) was added and mixed before pouring onto 90mm NZY agar (see Appendix) plates and incubating overnight at 37°C. Dilutions which gave 30-300 plaques/plate were used to calculate the titre of the library as plaque forming units (pfu)/µl.



### 2.7.3 Sizing of cDNA inserts after mass excision

In order to check the insert quality of the cDNA library, a 1µl aliquot of the library was used in the Uni-ZAP™ XR excision procedure (section 2.7.7). A number of single, well-isolated colonies were selected at random and the derived pBluescript plasmid of each was isolated using a QIAprep Spin Plasmid Miniprep kit (Qiagen, UK) and digested with *Eco*RI and *Xho*I as described in section 2.5.6.1 to isolate the cDNA inserts. A 1µg aliquot of the digest was used in minigel analysis as described in section 2.5.3.2. The average cDNA insert size could then be estimated.

### 2.7.4 Primary screening

Aliquots of the library were chosen at random and from each a sample containing approximately 50000 pfu was mixed with 600µl of OD<sub>600</sub>=0.5 host XL-1 Blue cells in a Falcon tube. These were incubated with shaking at 37°C for 25 minutes, then mixed with 7ml of pre-heated (50°C) top agarose (see Appendix) prior to being poured onto 150mm NZY agar plates and incubated for 6-8 hours at 37°C. As soon as plaques of approximately 1mm diameter were visible the plates were removed and stored at 4°C for at least 2 hours before blotting to prevent subsequent adhesion of agar to the nylon blotting filters.

### 2.7.5 Colony hybridisation

Colony hybridisations were performed essentially as described in the Hybond™-N<sup>+</sup> protocol (Amersham, UK). Plates were removed from storage at 4°C and a 145mm Hybond™-N<sup>+</sup> filter placed onto the surface of the agar. Using a sterile needle the orientation of the filter with the agarose was



marked. Duplicate filters were used for each plate, the first remaining on the plate for 1 minute and the second for 2 minutes. Each filter was then placed, colony side up, on a pad of Whatman® 3MM paper (Whatman, UK) soaked in denaturing solution (1.5M sodium chloride, 500mM sodium hydroxide) for 7 minutes. Filters were then transferred to a fresh 3MM pad soaked in neutralising solution (1.5M sodium chloride, 500mM Tris-HCl (pH 7.2), 1mM Na<sub>2</sub>EDTA) for 3 minutes. This neutralising step was repeated using a fresh 3MM pad soaked in neutralising solution. The filters were then washed briefly in 2x SSPE (see Appendix) and placed on a pad of 3MM paper soaked in 0.4M sodium hydroxide for 20 minutes. Following this, the filters were washed briefly in 5x SSPE before hybridisation.

Hybridisation of the colony blots and autoradiography were performed exactly as for Southern blots (sections 2.5.6 and 2.5.7)

## 2.7.6 Secondary and Tertiary Screening

Autoradiographs containing plaques of interest were orientated, first against the corresponding master filters then, after marking, against the agar plates from which the filters had originated in order to identify positive plaques. Each positive plaque was then cored from the agar using the wide end of a sterile Pasteur pipette and transferred to a sterile microfuge tube containing 500µl of SM buffer (100mM sodium chloride, 50mM Tris-HCl (pH 7.5), 0.2% (w/v) magnesium sulphate (7-hydrate), 2% gelatin) and 20µl chloroform. The tube was then vortexed to release the phage particles from the agar and incubated at 4°C overnight.

These isolated plaques were then replated on 90mm NZY agar plates using 1µl of the following dilutions (in SM buffer) of the cored-out plaque stocks: 1:1000, 1:5000 and 1:10000. A 1µl aliquot of diluted phage was then incubated with 200µl of OD<sub>600</sub>=0.5 XL-1 Blue host cells and plated onto

90mm NZY agar plates in 3.5ml of top agarose as described in section 7.2.2. Plates showing 50-100 well-isolated single plaques were then selected for colony hybridisation as described above. From this second round of screening, two or three well-isolated positive plaques from each plate were cored out and stored at 4°C. Where necessary, a third round of screening was performed in order to isolate single positive plaques.

### 2.7.7 *In vivo* excision of pBluescript phagemids

The *in vivo* excision of pBluescript phagemids from positive plaques was performed using the ExAssist/SOLR system. The ExAssist helper phage contains an amber mutation that prevents replication of the phage genome in a nonsuppressing *E.coli* strain such as SOLR, thus eliminating problems associated with helper phage co-infection.

Cultures of SOLR and XL-1 Blue cells were prepared in 10ml LB Broth (see Appendix) and 10ml LB Broth containing 10mM magnesium sulphate and 0.2% maltose respectively and incubated overnight at 30°C with shaking. The cultures were diluted 1:100 in the same media and incubated at 37°C with shaking until the XL-1 Blue cells reached  $OD_{600}=0.2-0.5$ . These were then pelleted at 3000g for 5 minutes at room temperature and resuspended in 10mM magnesium sulphate at  $OD_{600}=1.0$ . Meanwhile, the SOLR cells were allowed to reach  $OD_{600}=0.5-1.0$  before being removed and incubated at room temperature until ready for use.

The following were then combined in a Falcon tube for each plaque to be excised:-

200µl	$OD_{600}=1.0$ XL-1 Blue cells
10µl	phage stock (containing $> 1 \times 10^5$ pfu)
1µl	ExAssist helper phage (containing $> 1 \times 10^6$ pfu)

A control mixture of XL-1 Blue cells and helper phage was also set up. Each tube was incubated at 37°C for 25 minutes with shaking before adding 3ml LB broth and incubating for 2-2.5 hours at 37°C with shaking. The tubes were then centrifuged at 2000g for 15 minutes at room temperature and the supernatant transferred to a sterile tube before being incubated at 70°C for 15 minutes. The tubes were then centrifuged at 4000g for 15 minutes at room temperature and the supernatant decanted to a sterile tube. This tube contained the excised pBluescript phagemid packaged as filamentous phage particles and could be stored at 4°C for 1-2 months.

For each rescued phagemid, 200µl of the previously prepared SOLR cells were added to each of 2 sterile microfuge tubes, one containing 10µl of the phagemid stock and the second containing 100µl of the phagemid stock. The tubes were then incubated at 37°C for 25 minutes with shaking. The pBluescript plasmid contains the ampicillin resistance gene Amp<sup>r</sup>, hence selection of SOLR cells containing the pBluescript plasmid was achieved by plating 10-50µl from each tube onto LB agar plates (see Appendix) containing 50µg ml<sup>-1</sup> ampicillin and incubating overnight at 37°C.

#### 2.7.8 Long-term storage of bacterial cultures

Glycerol stocks for long-term storage of bacterial cultures were prepared as described in Sambrook *et al.* (1989). A 5ml LB broth (see Appendix) culture containing the appropriate selective antibiotic (see Appendix) was inoculated with a single bacterial colony and incubated for 16-20 hours at 37°C with shaking. The following day 850µl of the culture was added to each of 4-5 sterile microfuge tubes containing 150µl of sterile glycerol and vortexed. The glycerol stocks were then flash-frozen in liquid nitrogen and stored at -80°C.

## 2.7.9 Analysis of positive clones using PCR

The cDNA inserts contained within putative positive clones could be amplified by PCR using primers designed from the flanking 5' and 3' pBluescript M13 regions. Subsequent Southern analysis (as described in sections 2.5.5 to 2.5.7) allowed the distinction between true positive (hybridising) and false positive (non-hybridising) clones at an early stage of screening and also allowed an estimation of their size.

### 2.7.9.1 PCR amplification of cDNA inserts from plaques

PCR of cDNA inserts from phage stocks was performed essentially as described by Clackson *et al.* (1991). The following were combined in a sterile 0.2ml PCR tube:-

14.00µl	sterile distilled water	
2.00µl	10x Taq buffer (+ MgCl <sub>2</sub> )	(Promega, UK)
1.00µl	1:50 dilution of phage stock	
0.25µl	M13 forward primer (20mM)	(Cruachem, UK)
0.25µl	M13 reverse primer (20mM)	(Cruachem, UK)
2.00µl	nucleotide mix (1.25mM each base)	(Pharmacia, UK)
0.25µl	Taq polymerase (5U µl <sup>-1</sup> )	(Promega, UK)

The following program was then carried out in an MJR PTC-100™ thermal cycler (Genetic Instrumentation, UK):-

Program 1 (1 cycle, linked to program 2)

95°C 5 minutes; 55°C 2 minutes; 72°C 3 minutes

Program 2 (25 cycles, linked to program 3)

95°C 1 minute; 55°C 2 minutes; 72°C 3 minutes

Program 3 (1 cycle)

95°C 1 minute; 55°C 2 minutes; 72°C 15 minutes

#### *2.7.9.2. PCR amplification of cDNA inserts from bacterial colonies*

The cDNA inserts of plasmids contained in bacterial colonies were PCR amplified using the method described in 2.7.9.1 except that the aliquot of phage stock was replaced by sterile distilled water and, using a sterile toothpick, the edge of a single bacterial colony was used to inoculate the 20µl PCR mix.

## 2.8 PARTIAL SEQUENCING OF ISOLATED CLONES

### 2.8.1 Plasmid preparation

Plasmid was isolated for sequencing using the QIAprep Spin Plasmid™ Miniprep kit (Qiagen, UK) as previously described (section 2.5.6.1). A 3-5µg aliquot of the plasmid was precipitated by the addition of 0.1 volumes of 3M sodium acetate (pH 6.0) and 2 volumes of ethanol, then incubated at -80°C for 15 minutes. The plasmid was then pelleted at 10000g for 10 minutes at 4°C, washed with 70% (v/v) ethanol and denatured ready for sequencing by resuspending in 50µl of 0.2M sodium hydroxide, 0.2mM Na<sub>2</sub>EDTA and incubating at 37°C for 30 minutes. Precipitation of the DNA was carried out by the addition of 2 volumes of ethanol and 0.1 volume of 3M sodium acetate (pH 5.2) and incubation at -80°C for 15 minutes. The sample was then centrifuged at 10000g for 10 minutes at room temperature, the pellet washed with 70% (v/v) ethanol, vacuum-dried, then resuspended in 7µl sterile distilled water.

### 2.8.2 Sequencing reactions

These were carried out using the Sequenase™ version 2.0 kit (United States Biochemical, USA) according to the manufacturers instructions. The kit utilises the dideoxy chain termination method of Sanger *et al* (1977). Reactions were carried out using primers designed for the M13 forward and M13 reverse regions flanking the 5' and 3' ends of each cDNA insert.

### 2.8.2.1 Annealing reaction

In a microfuge tube the following were combined:-

1 $\mu$ l	Primer (3 $\mu$ g $\mu$ l <sup>-1</sup> )
2 $\mu$ l	10x Sequenase Reaction Buffer
7 $\mu$ l	Plasmid preparation (3-5 $\mu$ g)

The sample was heated at 65°C for 2 minutes then allowed to cool to room temperature over a period of 30 minutes before being placed on ice. Meanwhile, 4 microfuge tubes each had 2.5 $\mu$ l of either the ddGTP, ddATP, ddTTP or ddCTP termination mix (each containing 80 $\mu$ M dGTP, 80 $\mu$ M dATP, 80 $\mu$ M dTTP, 80 $\mu$ M dCTP, 50mM sodium chloride and 8 $\mu$ M of the appropriate dideoxy nucleotide) added then were pre-warmed at 37°C until needed.

### 2.8.2.2 Labelling reaction

Labelling mix (7.5 $\mu$ M dGTP, 7.5 $\mu$ M dATP, 7.5 $\mu$ M dTTP) was diluted 1:5 in sterile distilled water and Sequenase version 2.0 (USB, USA) was diluted 1:8 in Sequenase dilution buffer. The following were then added to the annealed template primer reaction:-

1 $\mu$ l	0.1M DTT
2 $\mu$ l	dilute labelling mix
0.5 $\mu$ l	<sup>35</sup> S dCTP (37TBq mMole <sup>-1</sup> ; ICN, UK)
2 $\mu$ l	dilute Sequenase V2.0

The sample was mixed, taking care not to introduce bubbles and incubated for 5 minutes at room temperature.



### 2.8.2.3 Termination reaction

At the end of the labelling period a 3 $\mu$ l aliquot of the dGTP mix was transferred to the tube containing the ddGTP termination mix and incubation continued at 37°C for 5 minutes before 4 $\mu$ l of stop solution (95% (v/v) formamide, 20mM EDTA, 0.05% (v/v) xylene cyanol FF) was added. This procedure was repeated for each of the other three labelling reactions by adding to the corresponding termination mix tubes and all four completed sequencing reactions were then stored at -20°C until required for electrophoresis.

### 2.8.3 Polyacrylamide gel electrophoresis

Sequencing reactions were loaded onto a gel containing 6% (w/v) acrylamide, 0.3% (w/v) methylene-bisacrylamide and 7M urea made up in 1x TBE (see Appendix), using 1x TBE as the running buffer. The gel was run at a constant voltage of 1800V at 50°C. All samples were run in duplicate for 2 and 4 hours respectively. This allowed 250-300 bases to be sequenced at a time. After running, the gel was vacuum dried onto Whatman® 3MM paper (Whatman, UK) then exposed to Kodak X-OMAT™ (Kodak, UK) film overnight at room temperature in an autoradiographic cassette.

### 2.8.4. Bioinformatics

All DNA database sequence homology searches were performed using the BLASTN program of the University of Wisconsin GCG v8.1-UNIX sequence analysis software package. Sequence comparisons were performed using the BESTFIT program of the University of Wisconsin GCG v8.1-UNIX sequence analysis software package.



## 2.9 PHOTOGRAPHY

All autoradiographs and immunoblots were scanned into an Apple Macintosh PowerPC using a UMAX SuperVISTA S-12 scanner, then either printed onto film using the Adobe Photoshop software 3.0 application package or laserprinted using an Apple Macintosh Personal Laserwriter.

## CHAPTER 3

### Environmental Control of Leaf Nitrite Reductase Transcript in Barley

### 3.1 INTRODUCTION

Nitrate and light influence the development of nitrite reductase activity. Whether nitrate or nitrite, produced as a result of nitrate reduction, is the true inducer of nitrite reductase is unclear. Nitrite induces nitrite reductase activity in *Lemna minor* when present at 5mM, but nitrite reductase activity decreases at higher nitrite concentrations, followed by plant death within a few days (Joy, 1969). Unlike *Lemna minor* where only nitrite reductase activity was induced by nitrite, both nitrite and nitrate reductase activities increased in non-photosynthetic tobacco XD cell cultures exposed to nitrite (Chroboczek Kelker and Filner, 1971). However, nitrite was found to be toxic to XD cells above 1mM (Chroboczek Kelker and Filner, 1971), placing some doubt upon the *in vivo* role of nitrite as inducer for nitrite reductase. Use of the molybdenum analogue tungstate, a specific inhibitor of development of nitrate reductase activity (Wray and Filner, 1970), and hence of nitrite synthesis, did not inhibit development of nitrite reductase activity in response to nitrate (Chroboczek Kelker and Filner, 1971), suggesting that nitrate does not have to be reduced to nitrite to cause an increase in nitrite reductase activity. This is further supported by reports that barley mutants defective in nitrate reduction, hence unable to synthesise nitrite, possessed levels of nitrite reductase activity that were comparable with those found in wild-type control plants (Warner *et al*, 1977; Bright *et al*, 1983; Steven, 1986). The nitrate content of these nitrate reductase-deficient mutants were also comparable with nitrate levels found in wild-type control plants (Warner *et al*, 1977; Steven, 1986). Thus, in barley, it appears that nitrate is capable of increasing nitrite reductase activity directly, and not via its reduction to nitrite.

The interaction between nitrate and light in the regulation of nitrite reductase gene expression has been examined in white light-grown plants by

several workers. In the absence of both light and nitrate, nitrite reductase activity is low in mustard cotyledons (Rajasekhar and Mohr, 1986), pea leaves (Gupta and Beevers, 1983), rice leaves (Ogawa and Ida, 1987) and barley leaves (Duncanson *et al*, 1992) while high levels of nitrite reductase activity are found in plants treated with both light and nitrate (Gupta and Beevers, 1983; Rajasekhar and Mohr, 1986; Ogawa and Ida, 1987; Duncanson *et al*, 1992). In pea and barley, nitrate addition has no effect on leaf nitrite reductase activity in dark-grown plants (Gupta and Beevers, 1983), whereas nitrite reductase activity in dark-grown rice seedlings (Ogawa and Ida, 1987), or mustard (Rajasekhar and Mohr, 1986) exposed to nitrate is approximately 50% of the level found in light-grown plants. Light alone has no effect on the development of nitrite reductase activity in mustard (Schuster *et al*, 1987), suggesting that the light effect cannot act in the absence of nitrate and that nitrate is the true "inducer" of nitrite reductase activity.

Red/far red light pulse experiments in maize (Sharma and Sopory, 1984), mustard (Rajasekhar and Mohr, 1986), spinach (Seith *et al*, 1991), tobacco (Neininger *et al*, 1992) and barley (Seith *et al*, 1994) indicate the involvement of phytochrome in the light regulation of nitrite reductase. Red light treatment given up to 8 hours before nitrate addition can strongly enhance subsequent nitrate induction of nitrite reductase activity (Sharma and Sopory, 1984). The red light effect is reversed by far red light, but photoreversibility is reduced to zero within 2 hours. Sharma and Sopory (1984) argue that phytochrome induces a "biochemical signal" within 2 hours which can persist and cause enhancement of nitrate induction of nitrite reductase activity for a period of 8-12 hours. Signal storage is proposed to be a means of enabling the plant to maintain the appropriate levels of nitrite reductase (and indeed also of nitrate reductase) during the dark period of the natural light/dark cycle (Schuster *et al*, 1987).

Since photo-oxidative damage of the chloroplasts abolishes the action of nitrate and light on nitrite reductase activity development in mustard cotyledons, Rajasekhar and Mohr (1986) have postulated an indirect role for chlorophyll via plastid development to produce a "plastidic" signal. This signal is a pre-requisite for the induction of nitrite reductase by nitrate and light. However, nitrate and light (phytochrome) act independently of each other in controlling the appearance of nitrite reductase activity. Nitrate is the inducer proper, whereas phytochrome modulates the extent of the nitrate induced response.

Increases in nitrite reductase activity are correlated with increases in nitrite reductase cross-reacting material in wheat (Small and Gray, 1984), pea (Gupta and Beevers, 1984), rice (Ogawa and Ida, 1987) and barley (Duncanson *et al*, 1992). These results indicate that the environmentally-induced modulation of extractable nitrite reductase activity involves alteration of the enzyme level by *de novo* synthesis and is not mediated by a reversible activation-inactivation of the existing enzyme.

In leaves of white light-grown plants, up-regulation of nitrite reductase mRNA also requires a coaction of both light and nitrate such as in spinach (Back *et al*, 1988), maize (Lahners *et al*, 1988; Bowsher *et al*, 1991), tobacco (Faure *et al*, 1991) and birch (Friemann *et al*, 1992b). These results suggest that nitrate and light modulate the synthesis of leaf nitrite reductase mRNA in the plants studied, and this chapter describes the effect of light and nitrate on barley leaf nitrite reductase gene expression at the transcriptional level.

## 3.2 RESULTS

### 3.2.1 Extraction buffer tests

Studies of nitrite reductase and nitrate reductase activity levels have been performed in barley using different tissue extraction buffers, three of which were tested here. Leaf tissue from 6-day-old, white light-grown barley cv Golden promise plants treated with nitrate in the light for 18 hours was extracted with each of the tissue extraction buffers described by Kuo *et al* (1980), Ip *et al* (1990) and Small and Wray (1980), the compositions of which are described in Materials and Methods (Chapter 2), at a tissue:buffer ratio of 1:5 (g:ml). *In vitro* NADH-nitrate reductase and methyl viologen nitrite reductase activity assays were performed as described in Materials and Methods and average values are shown in Table 3.1. The tissue extraction buffer described by Kuo *et al* (1980) was found to give the highest specific activities for both the nitrite reductase and nitrate reductase assays and this buffer was chosen for use in all tissue extractions described in this work.

**Table 3.1:** Analysis of tissue extraction buffers

Extraction buffer	NADH-NR (nmoles nitrite produced/mg protein/h)	MV-NiR ( $\mu$ moles nitrite reduced/mg protein/h)
Kuo <i>et al</i> (1980)	$181 \pm 11$	$7.76 \pm 0.39$
Ip <i>et al</i> (1990)	$123 \pm 8$	$5.98 \pm 0.32$
Small and Wray (1980)	$148 \pm 7$	$6.90 \pm 0.29$

*In vitro* NADH-nitrate reductase activity (NADH-NR) and *in vitro* methyl viologen nitrite reductase (MV-NiR) activity in leaf tissue of 7-day-old, green barley cv Golden Promise plants grown in the absence of nitrate, then extracted with the buffers described by Ip *et al* (1990), Kuo *et al* (1980) and Small and Wray (1980) after treatment with 25mM potassium nitrate in the light for 18 hours. Plant growth, tissue extraction and *in vitro* enzyme assays were performed as described in Materials and Methods. Two independent experiments were performed in triplicate and the data from one of these experiments is shown.

### 3.2.2 Effect of light and nitrate on nitrite reductase gene expression

Six-day-old, white light-grown barley cv Golden Promise plants were either transferred to the dark or maintained in the light and either treated with half-Hoaglands solution containing 25mM potassium nitrate or half-Hoaglands solution without nitrate. Leaf tissue samples were taken at 0, 3 and 18 hours after treatment with or without nitrate for enzyme activity assays (Table 3.2), immunoblot analysis (Figure 3.1a) and northern analysis (Figure 3.1b) as described in Materials and Methods (Chapter 2).

In the absence of nitrate *in vitro* methyl viologen nitrite reductase activity is present at low levels in leaf tissue of plants either maintained in the light or transferred to the dark (Table 3.2). However, plants transferred to the dark possessed slightly lower nitrite reductase activity 18 hours after transfer as compared to nitrite reductase activity in the same plants before transfer. After treatment with nitrate for 18 hours, leaf tissue from plants maintained in the light shows an approximate tenfold increase in nitrite reductase activity (Table 3.2) and there is also a slight increase in the nitrite reductase activity of leaf tissue from plants treated with nitrate for 18 hours in the dark (Table 3.2).

Nitrite reductase cross-reacting material (NiR-CRM) at 63kDa and nitrite reductase apoprotein gene (*nii*) transcript show a similar pattern of nitrate induction to that of nitrite reductase activity, that is, in the absence of nitrate, low levels of NiR-CRM and *nii* transcript at 2.3kb appear in leaf tissue (Figure 3.1). NiR-CRM levels in the leaves of nitrate-less plants transferred to the dark for 18 hours are undetectable (Figure 3.1). Leaf tissue from plants treated with nitrate in the light for 18 hours possess high levels of NiR-CRM and *nii* transcript and there is also a slight increase in NiR-CRM and *nii* transcript in leaf tissue from plants treated with nitrate in the dark for 18 hours (Figure 3.1).



Plants grown in the absence of nitrate have undetectable levels of nitrate in leaf tissue (Table 3.2). Nitrate accumulation occurs in the leaf tissue of plants grown in both the dark and light and is detectable within 3 hours of exposure to nitrate (Table 3.2).

**Table 3.2: Effect of light and nitrate on *in vitro* nitrite reductase activity in barley leaf tissue**

*In vitro* methyl viologen nitrite reductase activity (MV-NiR) and nitrate content of leaf tissue from 7-day-old, green barley cv Golden Promise plants, grown in the absence of nitrate, extracted with the buffer described by Kuo *et al* (1980) at 0, 3 and 18 hours after the treatments described below. Plant growth, tissue extraction, *in vitro* methyl viologen nitrite reductase assays and nitrate content determinations were performed as described in Materials and Methods. Three independent experiments were performed in triplicate and the data from one of these experiments is shown.

Treatments:

-L-N: Plants transferred to the dark and grown in the absence of nitrate

+L-N: Plants maintained in the light and grown in the absence of nitrate

-L+N: Plants transferred to the dark and treated with 25mM potassium  
nitrate

+L+N: Plants maintained in the light and treated with 25mM potassium  
nitrate

Growth conditions	Time	MV-NiR ( $\mu$ moles mitrite reduced/mg protein/hour)	Nitrate content ( $\mu$ moles nitrate/g fresh wt)
-L-N	0	$0.85 \pm 0.11$	$0 \pm 0$
	3	$0.98 \pm 0.12$	$0 \pm 0$
	18	$0.50 \pm 0.06$	$0 \pm 0$
+L-N	0	$0.96 \pm 0.15$	$0 \pm 0$
	3	$0.74 \pm 0.09$	$0 \pm 0$
	18	$0.94 \pm 0.13$	$0 \pm 0$
-L+N	0	$0.88 \pm 0.13$	$0 \pm 0$
	3	$0.96 \pm 0.12$	$1.4 \pm 0.3$
	18	$1.50 \pm 0.15$	$11.8 \pm 1.2$
+L+N	0	$0.85 \pm 0.10$	$0 \pm 0$
	3	$1.87 \pm 0.10$	$1.9 \pm 0.3$
	18	$7.89 \pm 0.29$	$13.4 \pm 1.5$

**Figure 3.1: Nitrate and light regulation of nitrite reductase cross-reacting material and nitrite reductase transcript**

Steady-state levels of (a) nitrite reductase cross-reacting material and (b) nitrite reductase (*nir*) transcript in the leaf tissue described in Table 3.2. Immunoblot analysis was performed as described in Materials and Methods. RNA extractions and northern analysis were performed as described in Materials and Methods, using the partial barley nitrite reductase cDNA, BNiR1 (Ward *et al*, 1996), as a probe (NiR). The ethidium bromide-stained agarose gel is also shown (EtBr) and band sizes are shown on the left.

Lanes :	1, -L-N, 0 hours	7, -L+N, 0 hours
	2, -L-N, 3 hours	8, -L+N, 3 hours
	3, -L-N, 18 hours	9, -L+N, 18 hours
	4, +L-N, 0 hours	10, +L+N, 0 hours
	5, +L-N, 3 hours	11, +L+N, 3 hours
	6, +L-N, 18 hours	12, +L+N, 18 hours

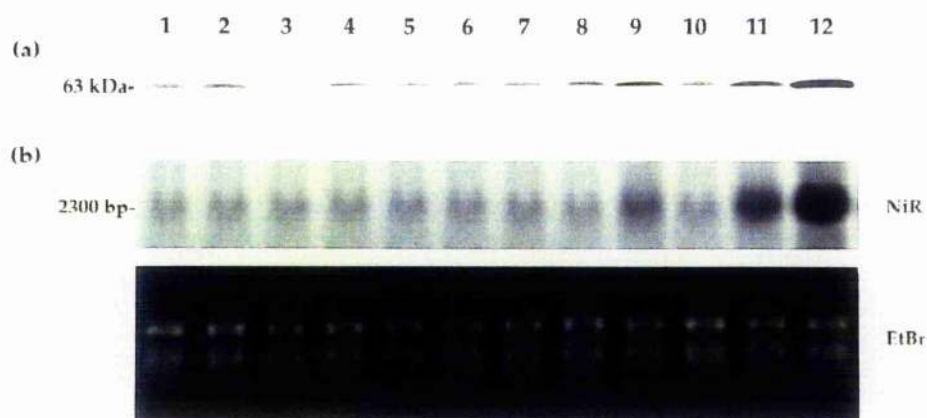
Abbreviations:

-L-N: Plants transferred to the dark and grown in the absence of nitrate

+L-N: Plants maintained in the light and grown in the absence of nitrate

-L+N: Plants transferred to the dark and treated with 25mM potassium  
nitrate

+L+N: Plants maintained in the light and treated with 25mM potassium  
nitrate



### 3.3 DISCUSSION

Increases in *in vitro* methyl viologen nitrite reductase activity in response to nitrate and light are predominantly due to *de novo* synthesis of nitrite reductase (*nii*) transcript (Figure 3.1), with concomitant increases in nitrite reductase cross-reacting material (Figure 3.1) and nitrite reductase activity (Table 3.2), and is not the result of some form of inactivation/activation mechanism of pre-existing enzyme molecules. Thus, regulation of nitrite reductase in barley leaf is similar to that reported in leaf tissue of wheat (Small and Gray, 1984) and pea (Gupta and Beevers, 1984) where increases in nitrite reductase activity correlated with increases in nitrite reductase cross-reacting material. Nitrite reductase transcript also shows a strong correlation with NiR-CRM and nitrite reductase activity (Figure 3.1; Table 3.2) with both nitrate and light required for high levels of nitrite reductase transcript (Figure 3.1). Thus it appears in barley that nitrate and light act predominantly at the transcriptional level.

Barley plants treated with nitrate in the dark were first exposed to light for 48 hours to allow normal maturation of chloroplasts and development of chlorophyll. Thus, the requirement for any "plastidic signal" due to chlorophyll development would be fulfilled. However, *in vitro* methyl viologen nitrite reductase activity, nitrite reductase cross-reacting material and nitrite reductase apoprotein transcript levels were greatly reduced up to 18 hours after treatment with nitrate in the dark as compared to those of leaves from plants treated with nitrate in the light (Table 3.2; Figure 3.1). In plants transferred to dark, *in vitro* leaf methyl viologen-nitrite reductase activity and leaf NiR-CRM levels decrease below the "basal" level observed before transfer (Table 3.2; Figure 3.1). Thus, the stored light signal described by Schuster *et al* (1987) for mustard cotyledons appears to have little effect in barley. The lack of such a system to regulate nitrite reductase activity levels

are unlikely to be detrimental to the plant since nitrite reductase is a relatively stable enzyme *in vivo*, with nitrite reductase activity reduced by only 28% in nitrate-grown plants where nitrate was subsequently withheld for 3 days (Gupta and Beevers, 1983). Therefore, the amount of nitrite reductase protein within the leaf tissue is likely to remain at an adequate level to reduce any nitrite, produced by nitrate reductase, to ammonium ions during the normal growth cycle of the plant.

The inability to synthesise high levels of nitrite reductase molecules in leaves of plants treated with nitrate in the dark is not due to the exclusion of nitrate as nitrate levels in leaf tissue from plants treated with nitrate in the light and in leaf tissue from plants treated with nitrate in the dark are similar (Table 3.2). Therefore, regulation of nitrite reductase under the environmental conditions tested is not mediated by the control of nitrate availability to leaf tissue.

## CHAPTER 4

### Characterisation of Barley Mutants Defective in Nitrite Reduction



## 4.1 INTRODUCTION

Study of the nitrate assimilation pathway at the genetic level in higher plants has been greatly facilitated by the analysis of nitrate assimilation mutants (reviewed in Chapter 1). However, most studies have been confined to higher plant mutants defective in nitrate uptake, identified on the basis of chlorate resistance or inability of individual plants to withdraw nitrate from the ambient medium, and mutants defective in nitrate reduction, identified on the basis of chlorate resistance, low *in vivo* nitrate reductase activity or inability to grow using nitrate as the sole nitrogen source (reviewed in Chapter 1). Mutants defective in nitrite reduction have been more difficult to isolate, probably due to the lack of an efficient selection method.

Higher plant mutants defective in nitrite reduction should be of use in dissecting this step at the genetic level as mutations in a number of loci would be likely to lead to a defect in nitrite reduction, such as within the nitrite reductase structural gene, affecting either catalytic activity or the proper functioning of the transit sequence required for chloroplast targeting; in the chloroplast envelope affecting recognition of the transit sequence and thus preventing import of the nitrite reductase protein into the chloroplast; in stromal proteinases; in prosthetic group synthesis; in transport of nitrite into the chloroplast (if this mechanism is protein mediated); in electron donation to nitrite reductase and in a component of the signal transduction pathway through which nitrate, light and the plastidic factor operate to regulate synthesis of nitrite reductase. However, mutations in some of these components of nitrite reduction could be lethal and unselectable.

On the basis that plants defective in nitrite reduction would lead to an *in vivo* accumulation of nitrite, Duncanson *et al* (1993) developed a selection method for isolating barley mutants. Barley seeds from a number of different cultivars were mutagenised with sodium azide, planted in field plots and M1

spikes carrying M<sub>2</sub> seed were collected (Duncanson *et al*, 1993). Three to five M<sub>2</sub> seed from a single M<sub>1</sub> spike were sown together in a single square in a numbered grid, where each grid contained fifty squares thus allowing up to 250 M<sub>2</sub> seed from 50 M<sub>1</sub> spikes to be assayed for leaf nitrite accumulation after treatment with nitrate in the light (Duncanson *et al*, 1993). The remaining M<sub>2</sub> seed from any M<sub>1</sub> spikes containing nitrite-accumulating M<sub>2</sub> seed could then be studied individually.

Using this procedure, Duncanson *et al* (1993) isolated eleven green nitrite-accumulating individuals from a population of 95000 M<sub>2</sub> barley plants, and these selected nitrite-accumulating individuals were then rescued from the vermiculite and grown in hydroponic culture using glutamine (1mM) as the sole nitrogen source. Although not all of these selections survived to flowering and some others that did were not fertile, one of these selections, STA3999 from the cultivar Tweed has been described (Duncanson *et al*, 1993) and is reviewed below.

STA3999 was crossed to the wild-type cultivar and the F<sub>2</sub> progeny were screened for leaf nitrite accumulation. Approximately one-quarter of the leaf tips excised accumulated nitrite and it was concluded that the nitrite-accumulating phenotype is due to a recessive mutation in a single nuclear gene, which was designated *Nir1*. Immunoblot analysis of individuals from the F<sub>2</sub> progeny showed that the nitrite reductase cross-reacting material (NiR-CRM) seen in nitrate-treated wild-type and nitrite non-accumulating F<sub>2</sub> and F<sub>3</sub> plants could not be detected in either the leaf or root of nitrite-accumulating F<sub>2</sub> and F<sub>3</sub> plants. *In vitro* nitrite reductase activity, measured with dithionite-reduced methyl viologen as the electron donor, in the leaf of nitrate-treated homozygous *nir1* mutant (nitrite-accumulating/NiR-CRM-minus) plants was greatly reduced, being approximately 10% of the activity found in the leaf of nitrite non-accumulating/NiR-CRM-plus siblings. *In vitro* NADH-nitrate reductase activity in homozygous *nir1* mutant plants

was approximately threefold higher than nitrite non-accumulating/NiR-CRM-plus siblings.

Although the homozygous *nir1* mutant could be maintained to flowering in liquid culture with either glutamine (1mM) or ammonium (2mM) as the sole nitrogen source, transfer of *nir1* mutants from nitrate-free conditions to compost resulted in plant death within 12 days of transfer, unlike nitrite non-accumulating/NiR-CRM-plus siblings treated under the same conditions which grew to maturity and flowered in a similar manner to the wild-type cultivar. Duncanson *et al* (1993) concluded that the *Nir1* locus determines the formation of nitrite reductase apoprotein in both the leaf and root of barley and speculated that it represents either the nitrite reductase apoprotein gene locus, or less likely, a regulatory locus whose product is required for the synthesis of nitrite reductase but not nitrate reductase. Elevation of NADH-nitrate reductase activity in the *nir1* mutant suggests a regulatory perturbation in the expression of the *Nar1* gene.

This chapter describes the characterisation of three further nitrite-accumulating barley selections, STA1010 and STA2760 from the wild-type cv Tweed and STA4169 from the wild-type cv Golden Promise and also the further study of the *nir1* mutant STA3999.

## 4.2 RESULTS

Analysis of the *nir1* mutant STA3999 has been performed using segregating F populations (Duncanson *et al*, 1993). Due to the difficulty in maintaining mutant selections in hydroponic culture, initial attempts to back-cross three further selections, STA1010 and STA2760 from cv Klaxon and STA4169 from cv Golden Promise, to their respective wild-type cultivars proved unsuccessful (J.L.Wray, unpublished), although back-crosses of these selections were later performed successfully by W.T.B. Thomas, SCRI, Invergowrie, UK, and F<sub>1</sub> seed were produced. Initial studies were performed using segregating M populations, derived from heterozygous M<sub>2</sub> plants rescued from M<sub>1</sub> spikes (J.L.Wray, unpublished). Biochemical studies were then repeated using F<sub>2</sub> populations of the selections when they became available, in order to confirm the conclusions reached during analysis of the M populations.

#### 4.2.1 Analysis of M population mutants

##### 4.2.1.1 *Inheritance of the nitrite accumulation phenotype within M populations of STA1010, STA2760 and STA4169*

Individual plants within segregating M<sub>5</sub> populations derived from STA1010 and STA2760 and within a segregating M<sub>4</sub> population derived from STA4169 were tested for leaf nitrite accumulation as described in Materials and Methods. In each segregating population approximately one-quarter of the leaf tips accumulated and excreted nitrite into the incubation medium, presumably as a consequence of a defect in *in vivo* nitrite reduction (Table 4.1). The progeny of one-half of the individuals in these segregating populations also segregated for the nitrite accumulation phenotype in the ratio one-quarter nitrite-accumulating (Acc<sup>+</sup>) to three-quarters nitrite non-accumulating (Acc<sup>-</sup>) (data not shown). It was concluded that nitrite accumulation in STA1010, STA2760 and STA4169 is due to a recessive mutation in a single nuclear gene.

**Table 4.1:** Inheritance of leaf nitrite accumulation within M populations from STA1010, STA2760 and STA4169

Population	Number of nitrite non- accumulators	Number of nitrite accumulators	$\chi^2$ (3:1)
M5 STA1010	277	90	0.04
M5 STA2760	202	69	0.03
M4 STA4169	236	74	0.20

Inheritance of leaf nitrite accumulation within segregating M<sub>5</sub> populations from STA1010 and STA2760 and within a segregating M<sub>4</sub> population from STA4169. Plant growth and leaf nitrite accumulation screens were performed as described in Materials and Methods. A  $\chi^2$  value of below 3.84 indicates that the segregation ratio is not significantly different at the 5% level from the Mendelian 3:1 ratio.

#### 4.2.1.2 Immunoblot analysis of STA1010, STA2760 and STA4169

Immunoblot analysis of total protein, using the polyclonal anti-barley nitrite reductase antibody (Duncanson *et al*, 1992), demonstrated that the leaf and root of wild-type barley plants and of  $\text{Acc}^-$  plants within segregating M5 populations derived from STA1010 and STA2760 and within a segregating M4 population derived from STA4169 possess nitrite reductase cross-reacting material (NiR-CRM) at 63kDa (Figure 4.1). However,  $\text{Acc}^+$  plants within the M populations were shown to have lost detectable NiR-CRM in their leaf and root (Figure 4.1).

Attempts to study the levels of nitrate reductase cross-reacting material (NR-CRM) in barley using a monoclonal anti-maize nitrate reductase antibody (Chérel *et al*, 1985) for immunoblot development were unsuccessful. For testing purposes, immunoblot analysis was performed on barley cv Golden Promise plants. Leaf tissue of plants grown in the absence of nitrate and from leaf and root tissue of plants treated with nitrate were analysed using concentrations of the antibody ranging from 1:500 to 1:50000 and immunoblots showed non-specific binding of the antibody to the protein. An example of an NR-CRM immunoblot is shown in Figure 4.2.

**Figure 4.1:** Nitrite reductase cross-reacting material in segregating M populations derived from STA1010, STA2760 and STA4169 and in wild-type barley

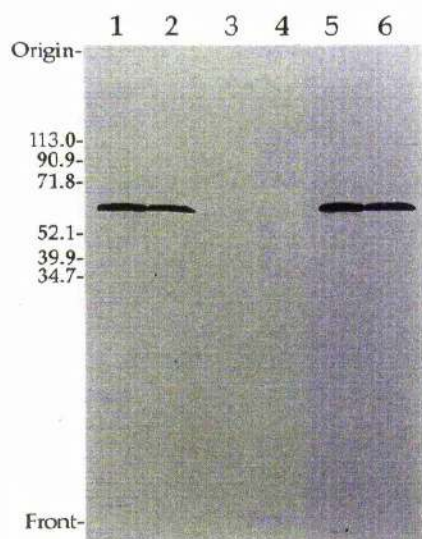
Immunoblot determination of nitrite reductase cross-reacting material in the leaf and root of 7-day-old, green plants, grown in the absence of nitrate, (a) within a segregating M<sub>5</sub> population from STA1010 and of the wild-type cv Klaxon, (b) within a segregating M<sub>5</sub> population from STA2760 and of the wild-type cv Klaxon and (c) within a segregating M<sub>4</sub> population from STA4169 and of the wild-type cv Golden Promise after extraction into the buffer described by Kuo *et al* (1980) after treatment with 25mM potassium nitrate in the light for 18 hours. Plant growth, leaf nitrite accumulation screens, tissue extraction and immunoblot analysis were performed as described in Materials and Methods. Marker sizes are shown on the left in kDa.

Lanes:

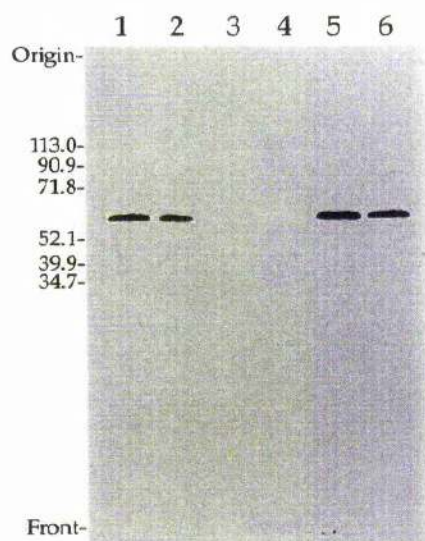
- 1, leaf from Acc<sup>-</sup> plant from a segregating M population
- 2, root from Acc<sup>-</sup> plant from a segregating M population
- 3, leaf from Acc<sup>+</sup> plant from a segregating M population
- 4, root from Acc<sup>+</sup> plant from a segregating M population
- 5, leaf from the wild-type cultivar
- 6, root from the wild-type cultivar



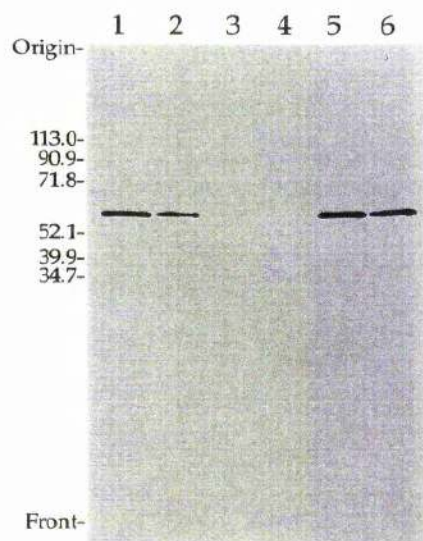
(a)



(b)



(c)

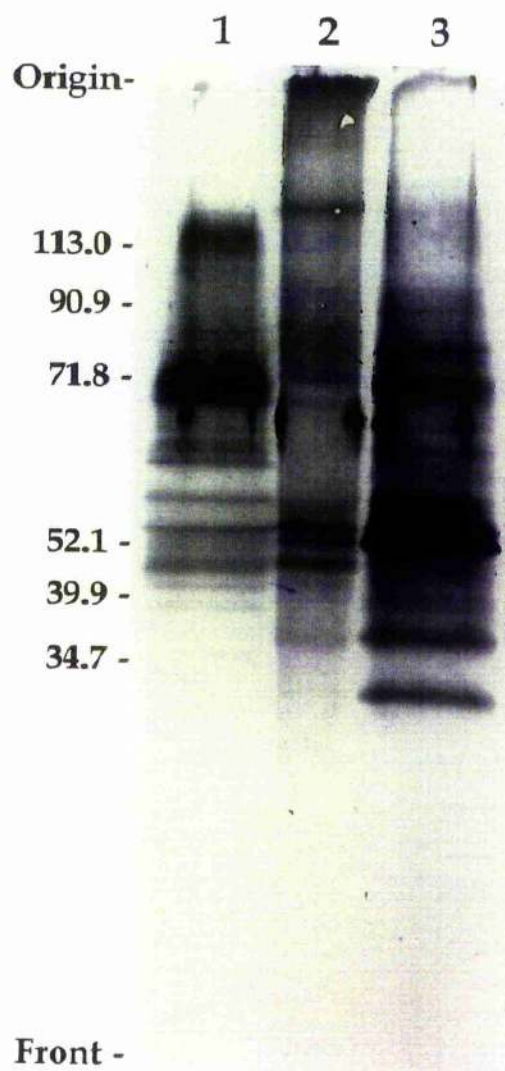


**Figure 4.2: Immunoblot determination of nitrate reductase cross-reacting material in wild-type cv Golden Promise**

Immunoblot determination of nitrate reductase cross-reacting material in the leaf and root of 7-day-old, green wild-type cv Golden Promise plants, grown in the absence of nitrate and extracted with the buffer described by Kuo *et al* (1980) after the treatments described below. Plant growth, tissue extractions and immunoblot analysis were performed as described in Materials and Methods except that an anti-maize nitrate reductase antibody (Chérel *et al*, 1985) was used at a dilution of 1:5000 in the development of the immunoblot. Marker sizes are shown on the left in kDa.

Lanes:

- 1, root from plant treated with 25mM potassium nitrate in the light for 18 hours
- 2, leaf from plant grown in the absence of nitrate
- 3, leaf from plant treated with nitrate in the light for 18 hours.



#### 4.2.1.3 Enzyme activities in STA1010, STA2760, STA4169 and STA3999

##### 4.2.1.3.1 Optimisation of *in vitro* NADH-nitrate reductase assay conditions

Previous studies involving *in vitro* NADH-nitrate reductase activity assays have demonstrated that the presence of NADH in the assay mix can inhibit the diazo coupling reaction and hence the colour development of the assay (Scholl *et al*, 1974), although this can be corrected by the addition of a termination solution, consisting of a 1:1 (v/v) mix of 1M zinc acetate and 0.3mM PMS, to the assay at a ratio of 0.2ml termination mix:1ml assay in order to remove the NADH from solution prior to the addition of the diazo-coupling reagents (Scholl *et al*, 1974).

For testing purposes, leaf tissue from barley cv Golden Promise plants treated with nitrate was extracted with the buffer described by Kuo *et al* (1980) and *in vitro* NADH-nitrate reductase assays were performed as described in Materials and Methods, except that a range of NADH concentrations and termination solution compositions were used (Table 4.2). NADH-nitrate reductase assays were performed using NADH concentrations ranging from 0.05mM to 1mM in the assay, either with or without the addition of 0.2ml of the assay termination solution described above (1:1 (v/v) mix of 1M zinc acetate and 0.3mM PMS) (Table 4.2a). The highest activity value was found using 0.2mM NADH in the assay, followed by the addition of the termination solution (Table 4.2a). *In vitro* NADH-nitrate reductase assays were also performed in order to determine whether increasing the final concentration of the termination solution would allow the use of a higher NADH concentration in the assay. Assays were performed using an NADH concentration of 0.2mM or 0.5mM, followed by the addition of 0.2ml of termination solutions ranging in composition from a 1:1 (v/v) mix of 1M zinc acetate and 0.3mM PMS to a 1:1 (v/v) mix of 2M

zinc acetate and 1mM PMS (Table 4.2b). In these assays, the highest activity value was found under the same conditions as previously (Table 4.2a). It was concluded that the highest activities would be found using a final concentration of 0.2mM NADH in the assay mix followed by the addition of 0.2ml of a 1:1 (v/v) mix of 1M zinc acetate and 0.3mM PMS to terminate the reaction and these conditions were used in all *in vitro* NADH-nitrate reductase assays in this chapter.

#### 4.2.1.3.2 *In vitro* methyl viologen nitrite reductase and *in vitro* NADH-nitrate reductase activities in STA1010, STA2760, STA4169 and STA3999

*In vitro* methyl viologen nitrite reductase and *in vitro* NADH-nitrate reductase activity assays were performed on the leaf tissue of plants within segregating M<sub>5</sub> populations derived from STA1010 and STA2760, within a segregating M<sub>4</sub> population of STA4169 and of the barley wild-type cultivars Klaxon and Golden Promise (Tables 4.3-4.5). Since the *in vitro* NADH-nitrate reductase assay conditions used here were different to those used in the characterisation of STA3999 (Duncanson *et al*, 1993), for comparative purposes NADH-nitrate reductase and methyl viologen nitrite reductase assays were also performed on leaf tissue of plants within a segregating F<sub>5</sub> population of STA3999 and from the wild-type barley cv Tweed under the same conditions described above (Table 4.6).

Leaf methyl viologen nitrite reductase activities of the Acc<sup>+</sup> plants within the four mutant populations STA1010, STA2760, STA4169 and STA3999 were similar in plants grown in the absence of nitrate and in plants treated with nitrate. These activities were 5-8% of those found in Acc<sup>-</sup> siblings and wild-type plants after treatment with nitrate and approximately 60% of those found in Acc<sup>-</sup> siblings and wild-type plants grown in the absence of nitrate (Tables 4.3-4.6).

When grown in the absence of nitrate, there was an approximate fourfold increase in the activity of leaf NADH-nitrate reductase in STA1010, STA2760 and STA3999  $\text{Acc}^+$  plants as compared to the  $\text{Acc}^-$  siblings and wild-type plants, whereas the leaf NADH-nitrate reductase activity in STA4169  $\text{Acc}^+$  plants was only approximately twofold higher than in the  $\text{Acc}^-$  siblings and wild-type plants (Tables 4.3-4.6). When treated with nitrate, leaf NADH-nitrate reductase activities were 2-2.5 times higher in the STA1010, STA2760 and STA3999  $\text{Acc}^+$  plants than in the  $\text{Acc}^-$  siblings and wild-type plants, whereas in the STA4169  $\text{Acc}^+$  plants the leaf NADH-nitrate activity was only 1.5-2 times higher than in the  $\text{Acc}^-$  siblings and wild-type plants (Tables 4.3-4.6).



**Table 4.2: Optimisation of *in vitro* NADH-nitrate reductase assay conditions**

Leaf tissue from 7-day-old, green wild-type cv Golden Promise, grown in the absence of nitrate, was treated with 25mM potassium nitrate in the light for 18 hours and extracted into the buffer described by Kuo *et al*, 1980). *In vitro* NADH-nitrate reductase assays were performed to test the following criteria:

(a) Effect of a termination solution on *in vitro* NADH-nitrate reductase activity measurements at different NADH concentrations in the NADH-nitrate reductase assay. NADH-nitrate reductase assays were performed as described in Materials and Methods except that NADH concentrations ranging from 0.05mM to 1mM were used in the assay and 0.2ml of the termination solution (consisting of a 1:1 (v/v) mix of 1M zinc acetate and 0.3mM PMS) was either added (+STOP) or withheld (-STOP).

(b) Effect of termination solution composition on *in vitro* NADH-nitrate reductase activity measurements at a higher NADH concentration. NADH assays were performed as described in Materials and Methods except that 0.2ml of termination (STOP) solutions of different compositions were added to assays containing either 0.2mM NADH or 0.5mM NADH. Termination solution compositions ranged from a 1:1 (v/v) mix of 1M zinc acetate and 0.3mM PMS to a 1:1 (v/v) mix of 2M zinc acetate and 1mM PMS.

Three independent experiments were performed in triplicate and the data from one of these experiments is shown.

(a)

NADH concentration (mM)	NADH-NR (nmoles nitrite produced/mg protein/h) (- STOP)	NADH-NR (nmoles nitrite produced/mg protein/h) (+ STOP)
0.05	114 ± 12	108 ± 8
0.10	182 ± 18	206 ± 13
0.20	154 ± 16	280 ± 12
0.50	95 ± 8	212 ± 15
1.00	55 ± 8	68 ± 6

(b)

STOP solution (mM) ZnAc/PMS	NADH-NR (nmoles nitrite produced/mg protein/h) (0.2mM NADH)	NADH-NR (nmoles nitrite produced/mg protein/h) (0.5mM NADH)
1M/0.3mM	264 ± 22	184 ± 15
1M/0.6mM	245 ± 19	206 ± 16
1M/1.0M	220 ± 11	180 ± 16
2M/0.3mM	250 ± 11	172 ± 9
2M/0.6mM	218 ± 22	112 ± 12
2M/1.0mM	203 ± 12	68 ± 5



**Table 4.3:** *In vitro* leaf enzyme activities and nitrate content within a segregating M<sub>5</sub> population from STA1010 and of the wild-type cv Klaxon

Growth conditions	Plant	NADH-NR (nmoles nitrite produced/ mg prot/h)	MV-NiR ( $\mu$ moles nitrite reduced/ mg prot/h)	Nitrate content ( $\mu$ mol/g fresh wt)
Nitrate untreated	Acc <sup>-</sup>	15.2 $\pm$ 1.3	0.83 $\pm$ 0.10	0 $\pm$ 0
	Acc <sup>+</sup>	51.3 $\pm$ 2.7	0.36 $\pm$ 0.11	0 $\pm$ 0
	Klaxon	14.8 $\pm$ 0.8	0.83 $\pm$ 0.20	0 $\pm$ 0
Nitrate treated	Acc <sup>-</sup>	277.7 $\pm$ 18.1	7.41 $\pm$ 0.88	13.6 $\pm$ 0.90
	Acc <sup>+</sup>	545.7 $\pm$ 22.6	0.38 $\pm$ 0.08	13.0 $\pm$ 0.77
	Klaxon	264.1 $\pm$ 12.2	7.62 $\pm$ 0.76	12.8 $\pm$ 0.60

*In vitro* NADH-nitrate reductase activity (NADH-NR), *in vitro* methyl viologen nitrite reductase activity (MV-NiR) and nitrate content of leaf tissue from 7-day-old, green plants, grown in the absence of nitrate, within a segregating M<sub>5</sub> population from STA1010 and of the wild-type cv Klaxon. Plants were either maintained in the absence of nitrate (nitrate untreated) or treated with 25mM potassium nitrate in the light for 18 hours (nitrate treated) and extracted with the buffer described by Kuo *et al* (1980). Plant growth, tissue extraction and enzyme assays were performed as described in Materials and Methods. Three independent experiments were performed and the data from one of these experiments is shown.

**Table 4.4:** Leaf enzyme activities and nitrate content within a segregating M5 population from STA2760 and of the wild-type cv Klaxon

Growth conditions	Plant	NADH-NR (nmoles nitrite produced/ mg prot/h)	MV-NiR ( $\mu$ moles nitrite reduced/ mg prot/h)	Nitrate content ( $\mu$ mol/g fresh wt)
Nitrate untreated	Acc <sup>-</sup>	14.7 $\pm$ 1.2	0.85 $\pm$ 0.07	0 $\pm$ 0
	Acc <sup>+</sup>	46.3 $\pm$ 3.1	0.41 $\pm$ 0.04	0 $\pm$ 0
	Klaxon	13.5 $\pm$ 2.1	0.98 $\pm$ 0.05	0 $\pm$ 0
Nitrate treated	Acc <sup>-</sup>	266.7 $\pm$ 18.1	7.58 $\pm$ 0.88	13.8 $\pm$ 1.6
	Acc <sup>+</sup>	533.6 $\pm$ 26.6	0.42 $\pm$ 0.09	12.1 $\pm$ 0.8
	Klaxon	253.4 $\pm$ 16.3	7.69 $\pm$ 0.61	13.2 $\pm$ 1.4

*In vitro* NADH-nitrate reductase activity (NADH-NR), *in vitro* methyl viologen nitrite reductase activity (MV-NiR) and nitrate content of leaf tissue from 7-day-old, green plants, grown in the absence of nitrate, from a segregating M5 population from STA2760 and from the wild-type cv Klaxon. Plants were either maintained in the absence of nitrate (nitrate untreated) or treated with 25mM potassium nitrate in the light for 18 hours (nitrate treated) and extracted with the buffer described by Kuo *et al* (1980). Plant growth, tissue extraction and enzyme assays were performed as described in Materials and Methods. Three independent experiments were performed and the data from one of these experiments is shown.

**Table 4.5:** Leaf enzyme activities and nitrate content within a segregating M<sub>4</sub> population from STA4169 and of the wild-type cv Golden Promise

Growth conditions	Plant	NADH-NR (nmoles nitrite produced/ mg prot/h)	MV-NiR (μmoles nitrite reduced/ mg prot/h)	Nitrate content (μmol/g fresh wt)
Nitrate untreated	Acc <sup>-</sup>	17.6 ± 2.1	0.75 ± 0.07	0 ± 0
	Acc <sup>+</sup>	32.4 ± 3.6	0.57 ± 0.03	0 ± 0
	GP	16.5 ± 1.1	0.88 ± 0.05	0 ± 0
Nitrate treated	Acc <sup>-</sup>	261.8 ± 21.1	7.61 ± 0.80	12.5 ± 1.7
	Acc <sup>+</sup>	466.9 ± 24.6	0.63 ± 0.08	12.6 ± 1.4
	GP	255.5 ± 16.9	7.50 ± 0.71	13.4 ± 1.0

*In vitro* NADH-nitrate reductase activity (NADH-NR), *in vitro* methyl viologen nitrite reductase activity (MV-NiR) and nitrate content of leaf tissue from 7-day-old, green plants, grown in the absence of nitrate, from a segregating M<sub>4</sub> population from STA4169 and from the wild-type cv Golden Promise. Plants were either maintained in the absence of nitrate (nitrate untreated) or treated with 25mM potassium nitrate in the light for 18 hours (nitrate treated) and extracted with the buffer described by Kuo *et al* (1980). Plant growth, tissue extraction and enzyme assays were performed as described in Materials and Methods. GP denotes the wild-type cv Golden Promise. Three independent experiments were performed and the data from one of these experiments is shown.

**Table 4.6:** Leaf enzyme activities and nitrate content within a segregating F<sub>5</sub> population from STA3999 and of the wild-type cv Tweed

Growth conditions	Plant	NADH-NR (nmoles nitrite produced/ mg prot/h)	MV-NiR ( $\mu$ moles nitrite reduced/ mg prot/h)	Nitrate content ( $\mu$ mol/g fresh wt)
Nitrate untreated	Acc <sup>-</sup>	14.5 $\pm$ 1.0	0.70 $\pm$ 0.05	0 $\pm$ 0
	Acc <sup>+</sup>	54.5 $\pm$ 2.8	0.41 $\pm$ 0.03	0 $\pm$ 0
	Tweed	12.3 $\pm$ 0.9	0.86 $\pm$ 0.10	0 $\pm$ 0
Nitrate treated	Acc <sup>-</sup>	275.7 $\pm$ 18.7	7.41 $\pm$ 0.62	12.8 $\pm$ 1.3
	Acc <sup>+</sup>	588.5 $\pm$ 36.4	0.36 $\pm$ 0.04	11.4 $\pm$ 0.9
	Tweed	266.8 $\pm$ 9.1	7.71 $\pm$ 0.65	13.2 $\pm$ 0.9

*In vitro* NADH-nitrate reductase activity (NADH-NR), *in vitro* methyl viologen nitrite reductase activity (MV-NiR) and nitrate content of leaf tissue from 7-day-old, green plants, grown in the absence of nitrate, from a segregating F<sub>5</sub> population from the cross STA3999  $\times$  Tweed and from the wild-type cv Tweed. Plants were either maintained in the absence of nitrate (nitrate untreated) or treated with 25mM potassium nitrate in the light for 18 hours (nitrate treated) and extracted with the buffer described by Kuo *et al* (1980). Plant growth, tissue extraction and enzyme assays were performed as described in Materials and Methods. Three independent experiments were performed and the data from one of these experiments is shown.

#### 4.2.1.4 *Growth characteristics of STA1010, STA2760 and STA4169*

When 7-day-old, green plants, grown in the absence of nitrate, within segregating M<sub>5</sub> populations derived from STA1010 (Figure 4.3a) and STA2760 (Figure 4.4a) and from a segregating M<sub>4</sub> population derived from STA4169 (Figure 4.5a) were transferred to compost in the light, the Acc<sup>-</sup> plants grew to maturity, flowered and set seed in a similar manner to wild-type plants. However, whilst Acc<sup>+</sup> plants produced new leaves, these began to wither from the tip downwards and subsequently the older leaves withered in the same manner. Withering could be detected within four days of transfer and within 12 days (STA1010 and STA2760; Figures 4.3b and 4.4b, respectively) or 14 days (STA4169; Figure 4.5b) of transfer the plants were dead.

**Figure 4.3: Growth of individuals within a segregating M<sub>5</sub> population from STA1010 and of the wild-type cv Klaxon**

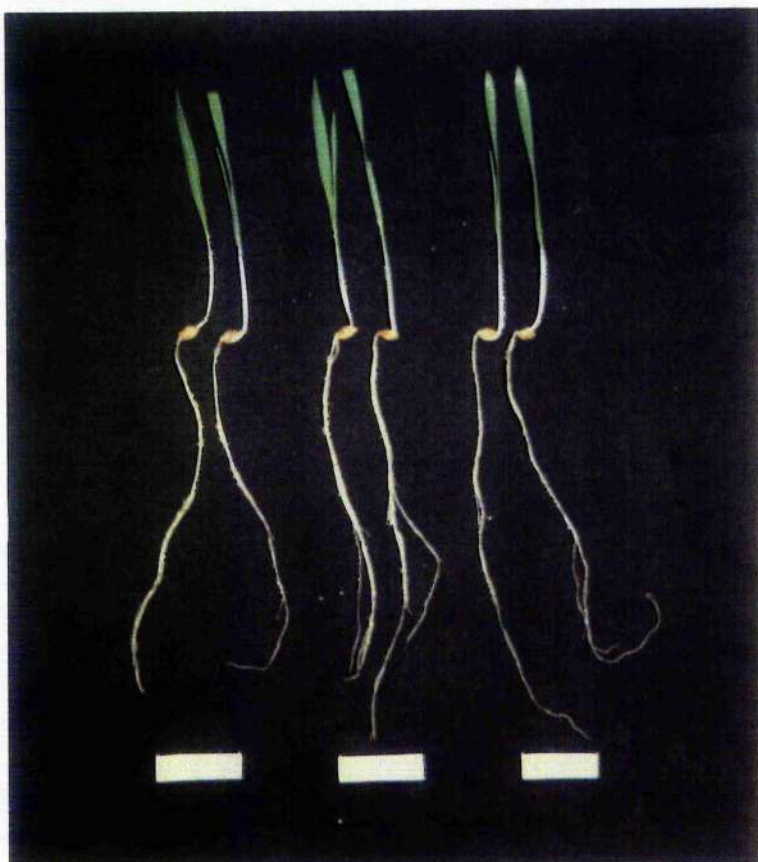
Plants were grown on nitrate-free vermiculite for (a) 7 days and photographed before transfer to compost. Plants were then photographed (b) 12 days after transfer.

(a) M<sub>5</sub> Acc<sup>-</sup> plants are shown on the *left* (average leaf length, 6.1cm); M<sub>5</sub> Acc<sup>+</sup> plants are shown in the *centre* (average leaf length, 6.3cm); plants of the wild-type cv Klaxon are shown on the *right* (average leaf length, 6.2cm).

(b) M<sub>5</sub> Acc<sup>-</sup> plants are shown on the *left* (average leaf length, 30.7cm); M<sub>5</sub> Acc<sup>+</sup> plants are shown in the *centre* (average leaf length, 12.6cm); plants of the wild-type cv Klaxon are shown on the *right* (average leaf length, 29.5cm).

Plant growth and leaf nitrite accumulation screens were performed as described in Materials and Methods.

(a)



(b)





**Figure 4.4:** Growth of individuals within a segregating M5 population from STA2760 and of the wild-type cv Klaxon

Plants were grown on nitrate-free vermiculite for (a) 7 days and photographed before transfer to compost. Plants were then photographed (b) 12 days after transfer.

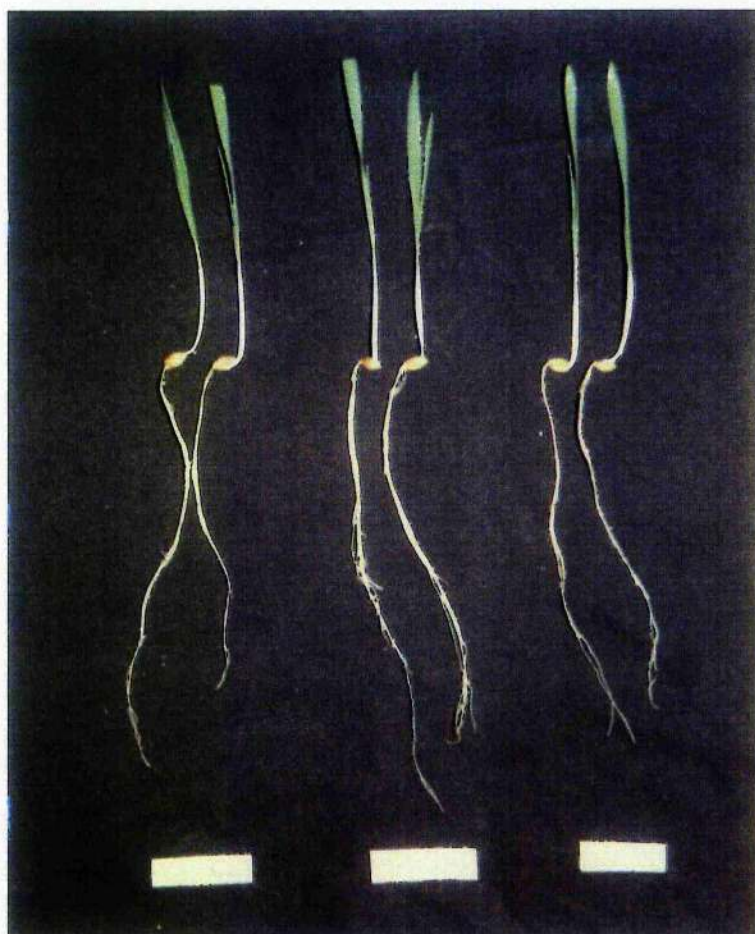
(a) M5 Acc<sup>-</sup> plants are shown on the *left* (average leaf length, 6.3cm); M5 Acc<sup>+</sup> plants are shown in the *centre* (average leaf length, 6.5cm); plants of the wild-type cv Klaxon are shown on the *right* (average leaf length, 6.4cm).

(b) M5 Acc<sup>-</sup> plants are shown on the *left* (average leaf length, 30.2cm); M5 Acc<sup>+</sup> plants are shown in the *centre* (average leaf length, 11.8cm); plants of the wild-type cv Klaxon are shown on the *right* (average leaf length, 28.5cm).

Plant growth and leaf nitrite accumulation screens were performed as described in Materials and Methods.



(a)



(b)



**Figure 4.5: Growth of individuals within a segregating M<sub>4</sub> population from STA4169 and of the wild-type cv Golden Promise**

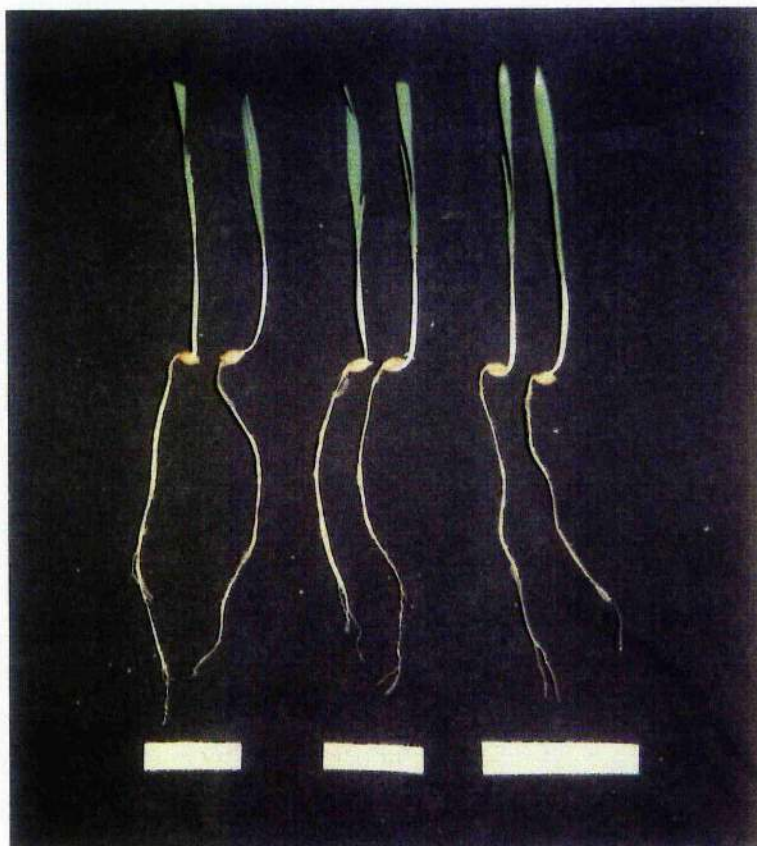
Plants were grown on nitrate-free vermiculite for (a) 7 days and photographed before transfer to compost. Plants were then photographed (b) 12 days after transfer.

(a) M<sub>4</sub> Acc<sup>-</sup> plants are shown on the *left* (average leaf length, 6.3cm); M<sub>4</sub> Acc<sup>+</sup> plants are shown in the *centre* (average leaf length, 6.4cm); plants of the wild-type cv Golden Promise are shown on the *right* (average leaf length, 6.7cm).

(b) M<sub>4</sub> Acc<sup>-</sup> plants are shown on the *left* (average leaf length, 32.1cm); M<sub>4</sub> Acc<sup>+</sup> plants are shown in the *centre* (average leaf length, 14.5cm); plants of the wild-type cv Golden Promise are shown on the *right* (average leaf length, 31.1cm).

Plant growth and leaf nitrite accumulation screens were performed as described in Materials and Methods.

(a)



(b)



#### 4.2.1.5 *In vivo* leaf nitrite accumulation in STA1010, STA2760 and STA4169

Accumulation of nitrite in the leaves of 7-day-old, green, nitrate-untreated NiR-CRM-minus plants within segregating M<sub>5</sub> populations derived from STA1010 and STA2760 and within a segregating M<sub>4</sub> population derived from STA4169, could be detected within 2 hours of exposure to 50mM potassium nitrate in the light (Figure 4.7). Nitrite accumulation in these plants continued over the course of the experiment, although nitrite levels in the STA4169 NiR-CRM-minus plants were lower than those in STA1010 and STA2760 NiR-CRM-minus plants after 18 hours of exposure to nitrate in the light (620nmol nitrite/g fresh wt, 920nmol nitrite/g fresh wt and 988nmol nitrite/g fresh wt respectively; Figure 4.6). NiR-CRM-plus plants within the segregating populations and wild-type plants did not accumulate nitrite after nitrate treatment (Figure 4.6).



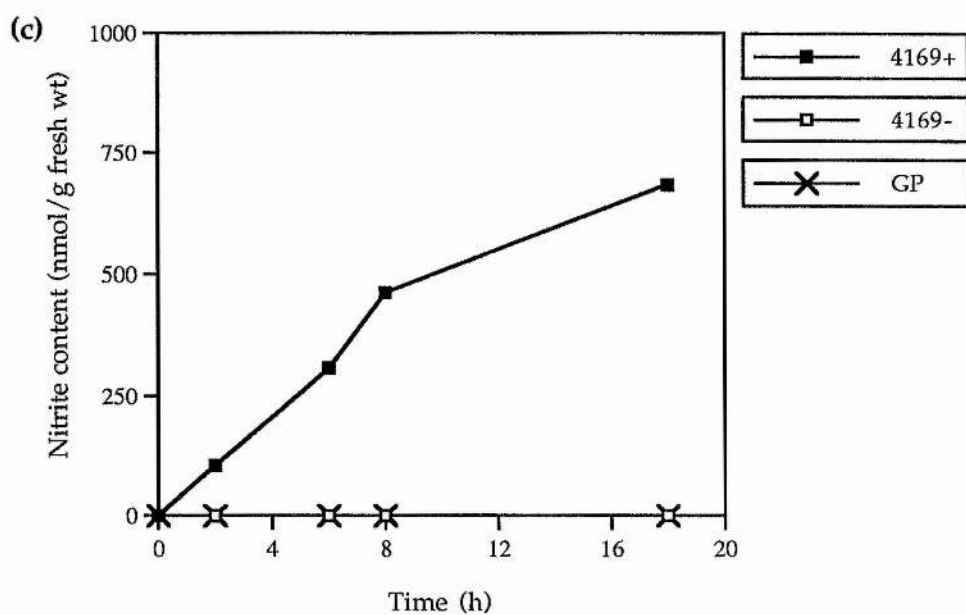
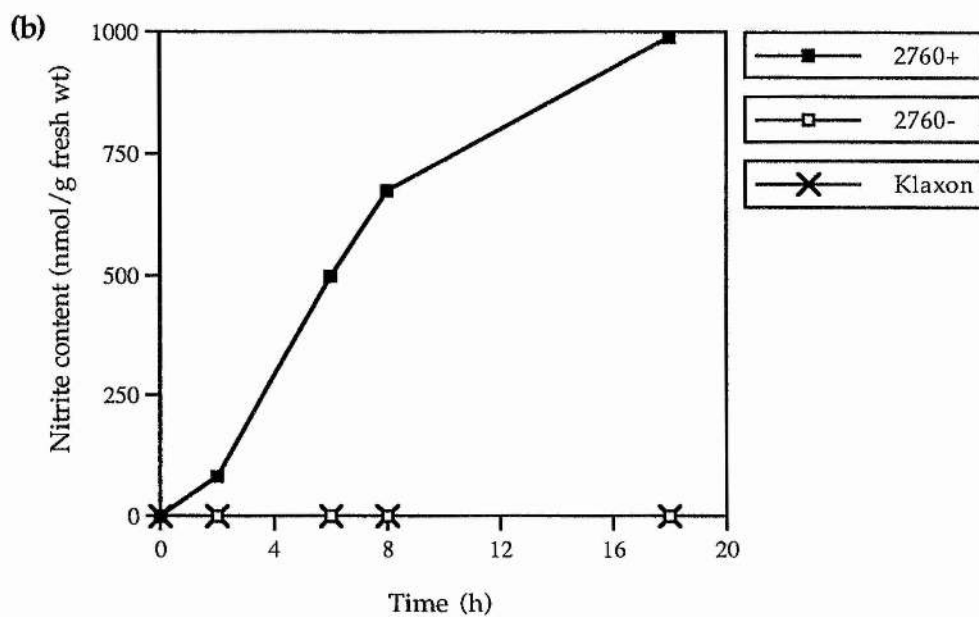
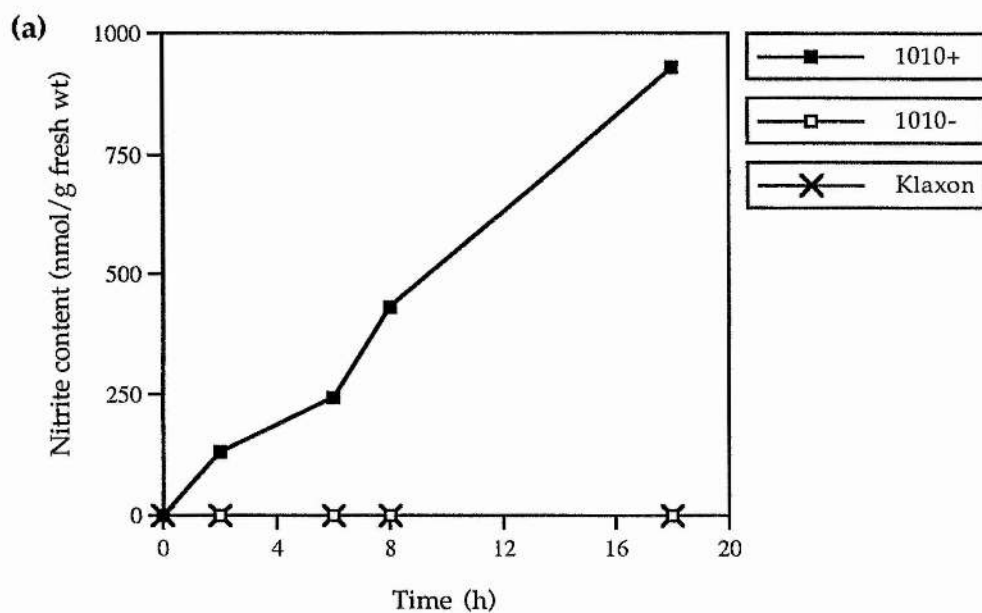
**Figure 4.6:** *In vivo* leaf nitrite accumulation within segregating M populations from STA1010, STA2760 and STA4169 and in wild-type barley

Seven-day-old, green plants, grown in the absence of nitrate, were treated with 50mM potassium nitrate in the light at 0 hours and leaf nitrite concentrations were determined at 0, 2, 6, 8 and 18 hours. Plant growth and leaf nitrite determinations were performed as described in Materials and Methods. Two independent experiments were performed in duplicate and the data from one of these experiments is shown.

(a) Plants within a segregating M<sub>5</sub> population from STA1010 and of the wild-type cv Klaxon. 1010- indicates plants from the M population which retain nitrite reductase cross-reacting material (NiR-CRM); 1010+ indicates plants from the M population which lack detectable NiR-CRM.

(b) Plants within a segregating M<sub>5</sub> population from STA2760 and of the wild-type cv Klaxon. 2760- indicates plants from the M population which retain nitrite reductase cross-reacting material (NiR-CRM); 2760+ indicates plants from the M population which lack detectable NiR-CRM.

(c) Plants within a segregating M<sub>4</sub> population from STA4169 and from of wild-type cv Golden Promise. 4169- indicates plants from the M population which retain NiR-CRM; 4169+ indicates plants from the M population which lack detectable NiR-CRM; GP indicates the wild-type cv Golden Promise.



#### 4.2.1.6 Allelism tests

Allelism tests were carried out between STA1010, STA2760 and STA4169. Since homozygous mutants ( $\text{Acc}^+$ ) are difficult to maintain to flowering, phenotypically wild-type  $\text{Acc}^-$  heterozygous plants, which carry a mutant allele, were used as the parents for crosses. In practice,  $\text{Acc}^-$  plants within segregating  $M_5$  populations from STA1010 and STA2760 were crossed to  $\text{Acc}^-$  plants within a segregating  $M_4$  population from STA4169 by W.T.B. Thomas, SCRI, Invergowrie, UK. These parents were then allowed to self and segregation for leaf nitrite accumulation was looked for in their progeny. Segregation of the progeny identified the parent plant as a heterozygous individual (Table 4.7). Crosses in which both parents were heterozygous for the mutant phenotype were studied further (Table 4.8).

If the mutations in STA1010, STA2760 and STA4169 are in the same gene then the mutant phenotype should be exhibited by one-quarter of the  $F_1$  progeny of crosses between heterozygous parents. If the mutations are in different genes then none of the  $F_1$  progeny of these crosses would be expected to exhibit the mutant phenotype. Of the 26  $F_1$  plants examined from the cross STA1010  $\times$  STA4169, 12 exhibited the mutant phenotype and of the 164  $F_1$  plants examined from the cross STA2760  $\times$  STA4169, 39 exhibited the mutant phenotype (Table 4.8) and it was concluded that STA1010, STA2760 and STA4169 contain mutations in the same gene. Similar studies have shown that STA1010 is defective in the same gene as STA3999 (J.L.Wray, unpublished), hence the data shows that STA1010, STA2760 and STA4169 are allelic to STA3999, therefore all four mutants are defective in the *Nir1* locus.

**Table 4.7: Inheritance of leaf nitrite accumulation in the self-pollinated progeny of allelism test parents**

The progeny resulting from the self-pollination of the  $\text{Acc}^- \text{M}_5$  individuals of STA1010 and STA2760 and the  $\text{Acc}^- \text{M}_4$  individuals of STA4169 used in cross-pollination experiments for allelism tests were grown and screened for leaf nitrite accumulation as described in Materials and Methods. A  $\chi^2$  value of below 3.84 indicates that the segregation ratio is not significantly different at the 5% level from the Mendelian 3:1 ratio.



Population	Parent number	Number of nitrite non-accumulators in progeny	Number of nitrite accumulators in progeny	$\chi^2$ (3:1)
M <sub>5</sub> STA1010	1010/10/10	12	2	0.86
	1010/10/18	12	6	0.67
M <sub>5</sub> STA2760	2760/7/3	10	4	0.10
	2760/7/12	7	6	3.10
	2760/7/13	8	6	2.39
	2760/7/15	7	4	0.77
M <sub>4</sub> STA4169	4169/8/2	7	5	1.78
	4169/8/6	23	5	0.76
	4169/8/21	8	7	3.76

**Table 4.8: Inheritance of the leaf nitrite accumulation phenotype in the F<sub>1</sub> progeny of crosses between allelism test parents**

The F<sub>1</sub> progeny resulting from cross-pollinations performed between the Acc<sup>-</sup> M<sub>5</sub> individuals of STA1010 and the Acc<sup>-</sup> M<sub>4</sub> individuals of STA4169 and between the Acc<sup>-</sup> M<sub>5</sub> individuals of STA2760 and the Acc<sup>-</sup> M<sub>4</sub> individuals of STA4169 described in Table 4.6 were grown and screened for leaf nitrite accumulation as described in Materials and Methods. A  $\chi^2$  value of below 3.84 indicates that the segregation ratio is not significantly different at the 5% level from the Mendelian 3:1 ratio.

Cross	Number of nitrite non- accumulators in F <sub>1</sub>	Number of nitrite accumulators in F <sub>1</sub>	$\chi^2$ (3:1)
1010/10/10 x 4196/8/2	11	5	0.29
1010/10/10 x 4169/8/6	10	4	0.10
1010/10/10 x 4169/8/21	7	3	0.13
TOTAL 1010 x 4169	26	12	0.88
2760/7/3 x 4169/8/2	16	3	0.86
2760/7/12 x 4169/8/6	19	4	0.61
2760/7/12 x 4169/8/21	8	3	0.03
2760/7/13 x 4169/8/2	28	6	0.99
2760/7/13 x 4169/8/6	11	7	1.86
2760/7/15 x 4169/8/2	11	8	2.96
2760/7/15 x 4169/8/6	14	4	0.08
2760/7/15 x 4169/8/21	18	4	0.56
TOTAL 2760 x 4169	125	39	0.13

## 4.2.2 Analysis of F population mutants

### 4.2.2.1 Isolation of F<sub>2</sub> populations of STA1010, STA2760 and STA4169

Due to the difficulty in maintaining homozygous *nir1* mutants to flowering, initial attempts to back-cross the mutants STA1010, STA2760 and STA4169 were unsuccessful (J.L.Wray, unpublished). However, further attempts to maintain the plants were successful and several independent back-crosses were performed between STA1010 and Klaxon, STA2760 and Klaxon, and STA4169 and Golden Promise by W.T.B Thomas at SCRI, Invergowrie, UK. F<sub>1</sub> seed were harvested, cultivated and allowed to self-pollinate to produce large populations of F<sub>2</sub> seed (Table 4.9). To confirm the conclusions thus far, biochemical studies which had previously been performed using the segregating M populations were repeated using these F<sub>2</sub> populations before further work was undertaken.

### 4.2.2.2 Inheritance of the nitrite accumulation phenotype within F<sub>2</sub> populations of STA1010, STA2760 and STA4169

Individual plants within the independent F<sub>2</sub> populations derived from the crosses STA1010 × Klaxon, STA2760 × Klaxon and STA4169 × Golden Promise were tested for leaf nitrite accumulation as described in Materials and Methods. In each population approximately one-quarter of the leaf tips accumulated and excreted nitrite into the incubation medium (Table 4.10). These observations were in agreement with the segregation analysis of the M populations described in section 4.2.1.1. That is, nitrite accumulation in STA1010, STA2760 and STA4169 is due to a recessive mutation in a single nuclear gene.

**Table 4.9: Isolation of F<sub>1</sub> populations from STA1010, STA2760 and STA4169**

Acc<sup>+</sup> individuals within segregating M<sub>5</sub> populations from STA1010 and STA2760 and within a segregating M<sub>4</sub> population from STA4169 were maintained in hydroponic culture and crossed to the wild-type cultivars from which they were isolated, using the wild-type cultivars as the female (pollen-recipient) parents and the number of F<sub>1</sub> seed produced is shown. Fourteen (\*) or thirteen (•) F<sub>1</sub> seed were then grown and allowed to self-pollinate to produce F<sub>2</sub> populations. Plant growth and leaf nitrite accumulation screens were performed as described in Materials and Methods. GP denotes the wild-type cv Golden Promise.

Cross	Female Parent (wild-type)	Male Parent (Acc <sup>+</sup> )	Number of F <sub>1</sub> seed produced
Klaxon x STA1010	Klaxon 1	1010/3	4
	Klaxon 2	1010/1 *	22
	Klaxon 3	1010/3 •	13
		1010/4 *	23
		1010/5 *	23
Klaxon x STA2760	Klaxon 1	2760/1 *	15
	Klaxon 2	2760/6	7
	Klaxon 3	2760/2	1
		2760/3	0
		2760/4 *	24
		2760/5 *	16
GP x STA4169	GP 1	4169/2 *	17
	GP 2	4169/2	6
		4169/4	1
	GP 3	4169/1 *	16
		4169/6	7
		4169/7 *	17

**Table 4.10: Inheritance of leaf nitrite accumulation within F<sub>2</sub> populations from STA1010, STA2760 and STA4169**

Population	Number of nitrite non- accumulators	Number of nitrite accumulators	$\chi^2$ (3:1)
F <sub>2</sub> STA1010	395	125	0.25
F <sub>2</sub> STA2760	366	124	0.02
F <sub>2</sub> STA4169	380	132	0.17

Inheritance of the leaf nitrite accumulation phenotype within F<sub>2</sub> populations from the crosses STA1010 x Klaxon, STA2760 x Klaxon and STA4169 x Golden Promise. Plant growth and leaf nitrite accumulation screens were performed as described in Materials and Methods. A  $\chi^2$  value of below 3.84 indicates that the segregation ratio is not significantly different at the 5% level from the Mendelian 3:1 ratio.

#### 4.2.2.3 Immunoblot analysis of STA1010, STA2760 and STA4169

Immunoblot analysis, using polyclonal anti-barley nitrite reductase antibody (Duncanson *et al*, 1992), of barley leaf tissue from plants within the F<sub>2</sub> populations derived from the crosses STA1010 x Klaxon, STA2760 x Klaxon and STA4169 x Golden Promise after treatment with nitrate showed that wild-type plants and Acc<sup>-</sup> plants within the F<sub>2</sub> populations possessed NiR-CRM at 63kDa in their leaf and root, unlike the Acc<sup>+</sup> plants which lacked detectable NiR-CRM in leaf and root (Figure 4.7).



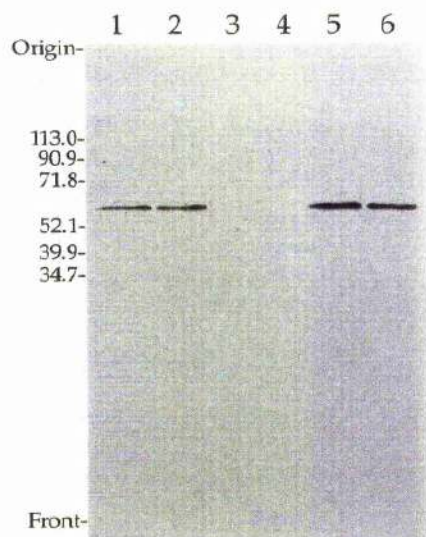
**Figure 4.7:** Nitrite reductase cross-reacting material within F<sub>2</sub> populations derived from STA1010, STA2760 and STA4169 and in wild-type barley

Immunoblot determination of nitrite reductase cross-reacting material in the leaf and root of 7-day-old, green plants, grown in the absence of nitrate, (a) within the F<sub>2</sub> population from the cross STA1010 x Klaxon and of the wild-type cv Klaxon, (b) within the F<sub>2</sub> population from the cross STA2760 x Klaxon and of the wild-type cv Klaxon and (c) within the F<sub>2</sub> population from the cross STA4169 x Golden Promise and of the wild-type cv Golden Promise which were extracted with the buffer described by Kuo *et al* (1980) after treatment with 25mM potassium nitrate in the light for 18 hours. Plant growth, leaf nitrite accumulation screens, tissue extraction and immunoblot analysis were performed as described in Materials and Methods. Marker sizes are shown on the left in kDa.

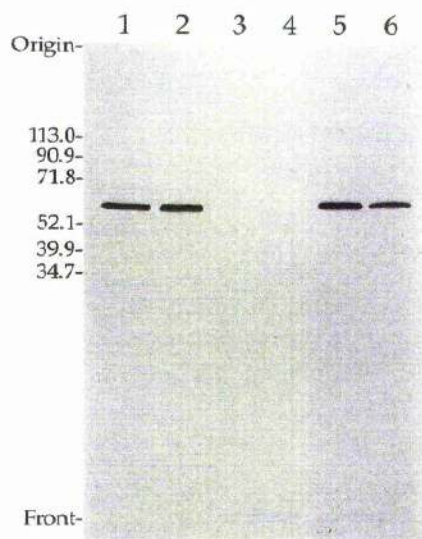
Lanes:

- 1, leaf from Acc<sup>-</sup> plant from the F<sub>2</sub> population
- 2, root from Acc<sup>-</sup> plant from the F<sub>2</sub> population
- 3, leaf from Acc<sup>+</sup> plant from the F<sub>2</sub> population
- 4, root from Acc<sup>+</sup> plant from the F<sub>2</sub> population
- 5, leaf from the wild-type cultivar
- 6, root from the wild-type cultivar

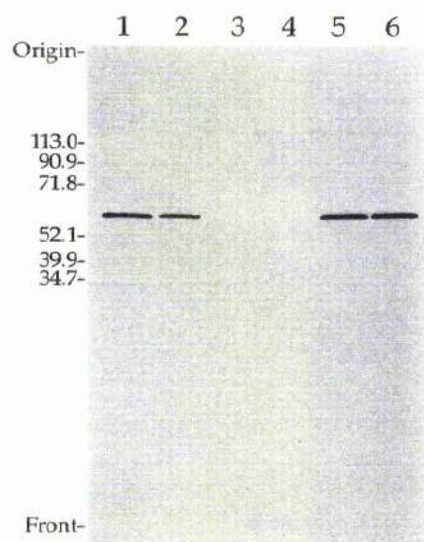
(a)



(b)



(c)



#### 4.2.2.4 *In vitro* enzyme activities in STA1010, STA2760, STA4169 and STA3999

*In vitro* methyl viologen nitrite reductase and *in vitro* NADH-nitrate reductase activity assays were performed on the leaf tissue of plants within the F<sub>2</sub> populations derived from the crosses STA1010 × Klaxon, STA2760 × Klaxon and STA4169 × Golden Promise and of the wild-type cultivars Klaxon and Golden Promise. As for the studies on M populations, plants within a segregating F<sub>5</sub> population of the cross STA3999 × Tweed and from the wild-type cv Tweed were used for comparative purposes. Also for comparative purposes, activity values from two independent experiments using the F populations, from which samples were also used for northern analysis, are shown in Tables 4.11-4.14.

Leaf methyl viologen nitrite reductase activities in the Acc<sup>+</sup> plants within the F populations of STA1010, STA2760, STA4169 and STA3999 were similar in plants grown in the absence of nitrate and in plants treated with nitrate (Tables 4.11-4.14). These activities were approximately 5-8% of those found in Acc<sup>-</sup> siblings and wild-type plants after treatment with nitrate and approximately 50% of those found in Acc<sup>-</sup> siblings and wild-type plants grown in the absence of nitrate (Tables 4.11-4.14).

When grown in the absence of nitrate, there was an approximate fourfold increase in the activity of leaf NADH-nitrate reductase in STA1010, STA2760 and STA3999 Acc<sup>+</sup> plants as compared to the activity in the Acc<sup>-</sup> siblings and wild-type plants, whereas in the STA4169 Acc<sup>+</sup> plants the leaf NADH-nitrate reductase activity was only approximately twofold higher than in the Acc<sup>-</sup> siblings and wild-type plants (Tables 4.11-4.14). When treated with nitrate, leaf NADH-nitrate reductase activities were approximately 2-2.5 times higher in the STA1010, STA2760 and STA3999 Acc<sup>+</sup> plants than in Acc<sup>-</sup> siblings and wild-type plants, whereas in the

STA4169  $\text{Acc}^+$  plants the activity was only approximately 1.5-2 times higher than in  $\text{Acc}^-$  siblings and wild-type plants (Tables 4.11-4.14).

Barley contains two nitrate reductase genes, *Nar1* and *Nar7* (Kleinhoffs *et al*, 1989). *Nar1* encodes the NADH-specific nitrate reductase which appears in the leaf and root of wild-type barley and *Nar7* encodes the NAD(P)H-bispecific nitrate reductase normally only found in the root (Kleinhoffs *et al*, 1989). In *Nar1* mutants, however, the *Nar7* gene is expressed in leaves (Dailey *et al*, 1982b) and to determine whether this is also the case in *Nir1* mutants, *in vitro* NADH-nitrate reductase and *in vitro* NADPH-nitrate reductase assays were performed, as described in Materials and Methods, on plants within the  $F_2$  populations derived from the crosses STA1010  $\times$  Klaxon, STA2760  $\times$  Klaxon and STA4169  $\times$  Golden Promise and on plants within a segregating  $F_5$  population derived from the cross STA3999  $\times$  Tweed. Assays were also performed on the wild-type cultivars from which these mutants were isolated (Klaxon, Klaxon, Golden Promise, Tweed respectively) under the same conditions and the results are shown in Table 4.15.

In plants grown in the absence of nitrate, leaf NADH-nitrate reductase activity is increased in the STA1010, STA2760, STA4169 and STA3999  $\text{Acc}^+$  plants as compared to the  $\text{Acc}^-$  siblings and wild-type plants (Table 4.15). Leaf NADPH-nitrate reductase activity, however, was absent in all plants tested (Table 4.15).

When treated with nitrate, leaf NADH-nitrate reductase activity was increased in STA1010, STA2760, STA4169 and STA3999  $\text{Acc}^+$  plants as compared to the  $\text{Acc}^-$  siblings and wild-type plants (Table 4.15). However, there does appear to be low levels of leaf NADPH-nitrate reductase activity in the nitrate-treated plants, although there is little difference in the activities between the  $\text{Acc}^+$  plants, the  $\text{Acc}^-$  siblings and the wild-type plants (Table 4.15). It is concluded that the higher levels of nitrate reductase activity in the four *nir1* mutants compared to the wild-type and  $\text{Acc}^-$  siblings are due to

higher levels of NADH-nitrate reductase and NAD(P)H-nitrate reductase makes no contribution to this effect.

**Table 4.11: Leaf enzyme activities and nitrate content within the F<sub>2</sub> population from STA1010 and of the wild-type cv Klaxon**

*In vitro* NADH-nitrate reductase activity (NADH-NR), *in vitro* methyl viologen nitrite reductase activity (MV-NiR) and nitrate content of leaf tissue from 7-day-old, green plants, grown in the absence of nitrate, within the F<sub>2</sub> population from the cross STA1010 x Klaxon and of the wild-type cv Klaxon. Plants were either maintained in the absence of nitrate (nitrate untreated) or treated with 25mM potassium nitrate in the light for 18 hours (nitrate treated) and extracted with the buffer described by Kuo *et al* (1980). Plant growth, tissue extraction and enzyme assays were performed as described in Materials and Methods.

Four independent experiments were performed in triplicate and the data from two of these experiments are shown here (1010/1 and 1010/2).

Growth conditions	Plant	NADH-NR (nmoles nitrite produced/ mg prot/h)	MV-NiR (μmoles nitrite reduced/ mg prot/h)	Nitrate content  (μmol/g fresh wt)
Nitrate untreated 1010/1	Acc <sup>-</sup>	17.4 ± 1.3	0.82 ± 0.05	0 ± 0
	Acc <sup>+</sup>	57.4 ± 4.6	0.46 ± 0.05	0 ± 0
	Klaxon	15.1 ± 2.0	0.79 ± 0.02	0 ± 0
Nitrate treated 1010/1	Acc <sup>-</sup>	294.6 ± 15.1	8.04 ± 0.40	13.1 ± 0.8
	Acc <sup>+</sup>	637.4 ± 31.2	0.34 ± 0.1	12.1 ± 1.1
	Klaxon	285.9 ± 28.1	7.40 ± 0.35	13.8 ± 0.5
Nitrate untreated 1010/2	Acc <sup>-</sup>	15.9 ± 2.1	0.88 ± 0.04	0 ± 0
	Acc <sup>+</sup>	62.3 ± 12.4	0.36 ± 0.02	0 ± 0
	Tweed	14.7 ± 1.1	0.70 ± 0.04	0 ± 0
Nitrate treated 1010/2	Acc <sup>-</sup>	286.3 ± 10.6	7.80 ± 0.30	14.2 ± 1.0
	Acc <sup>+</sup>	601.7 ± 27.1	0.40 ± 0.03	12.9 ± 0.9
	Tweed	272.4 ± 18.4	7.65 ± 0.51	13.6 ± 1.2

**Table 4.12: Leaf enzyme activities and nitrate content within the F<sub>2</sub> population from STA2760 and of the wild-type cv Klaxon**

*In vitro* NADH-nitrate reductase activity (NADH-NR), *in vitro* methyl viologen nitrite reductase activity (MV-NiR) and nitrate content of leaf tissue from 7-day-old, green plants, grown in the absence of nitrate, within the F<sub>2</sub> population from the cross STA2760 x Klaxon and of the wild-type cv Klaxon. Plants were either maintained in the absence of nitrate (nitrate untreated) or treated with 25mM potassium nitrate in the light for 18 hours (nitrate treated) and extracted with the buffer described by Kuo *et al* (1980). Plant growth, tissue extraction and enzyme assays were performed as described in Materials and Methods.

Four independent experiments were performed in triplicate and the data from two of these experiments are shown here (2760/1 and 2760/2).



Growth conditions	Plant	NADH-NR (nmoles nitrite produced/ mg prot/h)	MV-NiR ( $\mu$ moles nitrite reduced/ mg prot/h)	Nitrate content ( $\mu$ mol/g fresh wt)
Nitrate untreated 2760/1	Acc <sup>-</sup>	16.3 $\pm$ 0.8	0.94 $\pm$ 0.11	0 $\pm$ 0
	Acc <sup>+</sup>	59.8 $\pm$ 2.1	0.43 $\pm$ 0.05	0 $\pm$ 0
	Klaxon	14.5 $\pm$ 1.1	0.81 $\pm$ 0.10	0 $\pm$ 0
Nitrate treated 2760/1	Acc <sup>-</sup>	296.4 $\pm$ 15.2	8.50 $\pm$ 0.56	13.2 $\pm$ 0.6
	Acc <sup>+</sup>	606.6 $\pm$ 18.7	0.48 $\pm$ 0.06	12.1 $\pm$ 1.0
	Klaxon	271.6 $\pm$ 22.8	7.95 $\pm$ 0.71	12.8 $\pm$ 0.7
Nitrate untreated 2760/2	Acc <sup>-</sup>	18.1 $\pm$ 0.4	0.75 $\pm$ 0.03	0 $\pm$ 0
	Acc <sup>+</sup>	62.7 $\pm$ 2.5	0.32 $\pm$ 0.03	0 $\pm$ 0
	Klaxon	16.2 $\pm$ 1.7	0.66 $\pm$ 0.04	0 $\pm$ 0
Nitrate treated 2760/2	Acc <sup>-</sup>	283.1 $\pm$ 12.6	7.83 $\pm$ 0.26	12.4 $\pm$ 1.1
	Acc <sup>+</sup>	594.4 $\pm$ 38.1	0.38 $\pm$ 0.03	11.0 $\pm$ 0.8
	Klaxon	287.3 $\pm$ 21.7	7.60 $\pm$ 0.40	12.7 $\pm$ 1.3

**Table 4.13:** Leaf enzyme activities and nitrate content within the F<sub>2</sub> population from STA4169 and of the wild-type cv Golden Promise

*In vitro* NADH-nitrate reductase activity (NADH-NR), *in vitro* methyl viologen nitrite reductase activity (MV-NiR) and nitrate content of leaf tissue from 7-day-old, green plants, grown in the absence of nitrate, within the F<sub>2</sub> population from the cross STA4169 x Golden Promise and of the wild-type cv Golden Promise. Plants were either maintained in the absence of nitrate (nitrate untreated) or treated with 25mM potassium nitrate in the light for 18 hours (nitrate treated) and extracted with the buffer described by Kuo *et al* (1980). Plant growth, tissue extraction and enzyme assays were performed as described in Materials and Methods. GP denotes the wild-type cv Golden Promise.

Four independent experiments were performed in triplicate and the data from two of these experiments are shown here (4169/1 and 4169/2).

Growth conditions	Plant	NADH-NR (nmoles nitrite produced/ mg prot/h)	MV-NiR ( $\mu$ moles nitrite reduced/ mg prot/h)	Nitrate content ( $\mu$ mol/g fresh wt)
Nitrate untreated 4169/1	Acc <sup>-</sup>	18.2 $\pm$ 0.9	0.89 $\pm$ 0.08	0 $\pm$ 0
	Acc <sup>+</sup>	36.2 $\pm$ 1.1	0.37 $\pm$ 0.03	0 $\pm$ 0
	GP	15.6 $\pm$ 1.0	0.71 $\pm$ 0.05	0 $\pm$ 0
Nitrate treated 4169/1	Acc <sup>-</sup>	298.2 $\pm$ 12.8	7.82 $\pm$ 0.50	12.9 $\pm$ 0.8
	Acc <sup>+</sup>	556.0 $\pm$ 21.1	0.41 $\pm$ 0.05	10.6 $\pm$ 1.0
	GP	276.3 $\pm$ 18.6	7.13 $\pm$ 0.92	11.4 $\pm$ 0.9
Nitrate untreated 4169/2	Acc <sup>-</sup>	19.6 $\pm$ 1.6	0.85 $\pm$ 0.08	0 $\pm$ 0
	Acc <sup>+</sup>	40.0 $\pm$ 2.8	0.44 $\pm$ 0.08	0 $\pm$ 0
	GP	17.4 $\pm$ 0.8	0.80 $\pm$ 0.07	0 $\pm$ 0
Nitrate treated 4169/2	Acc <sup>-</sup>	277.4 $\pm$ 18.2	7.64 $\pm$ 0.40	11.8 $\pm$ 1.8
	Acc <sup>+</sup>	539.8 $\pm$ 29.5	0.50 $\pm$ 0.09	11.2 $\pm$ 0.7
	GP	268.8 $\pm$ 24.2	7.24 $\pm$ 0.56	10.9 $\pm$ 0.8

**Table 4.14:** Leaf enzyme activities and nitrate content within a segregating F<sub>5</sub> population from STA3999 and of the wild-type cv Tweed

*In vitro* NADH-nitrate reductase activity (NADH-NR), *in vitro* methyl viologen nitrite reductase activity (MV-NiR) and nitrate content of leaf tissue from 7-day-old, green plants, grown in the absence of nitrate, within a segregating F<sub>5</sub> population from the cross STA3999 × Tweed and of the wild-type cv Tweed. Plants were either maintained in the absence of nitrate (nitrate untreated) or treated with 25mM potassium nitrate in the light for 18 hours (nitrate treated) and extracted with the buffer described by Kuo *et al* (1980). Plant growth, tissue extraction and enzyme assays were performed as described in Materials and Methods.

Four independent experiments were performed in triplicate and the data from two of these experiments are shown here (3999/1 and 3999/2).

Growth conditions	Plant	NADH-NR (nmoles nitrite produced/ mg prot/h)	MV-NiR (μmoles nitrite reduced/ mg prot/h)	Nitrate content (μmol/g fresh wt)
Nitrate untreated 3999/1	Acc <sup>-</sup>	17.2 ± 1.5	0.80 ± 0.05	0 ± 0
	Acc <sup>+</sup>	62.5 ± 3.2	0.42 ± 0.05	0 ± 0
	Klaxon	15.4 ± 1.6	0.99 ± 0.04	0 ± 0
Nitrate treated 3999/1	Acc <sup>-</sup>	296.8 ± 12.7	7.34 ± 0.42	14.1 ± 1.4
	Acc <sup>+</sup>	630.6 ± 21.8	0.43 ± 0.04	13.6 ± 1.1
	Klaxon	291.4 ± 23.4	7.96 ± 0.61	13.8 ± 1.2
Nitrate untreated 3999/2	Acc <sup>-</sup>	15.1 ± 0.8	0.79 ± 0.08	0 ± 0
	Acc <sup>+</sup>	63.7 ± 5.8	0.38 ± 0.06	0 ± 0
	Klaxon	14.8 ± 0.7	0.94 ± 0.07	0 ± 0
Nitrate treated 3999/2	Acc <sup>-</sup>	276.7 ± 18.8	7.30 ± 0.71	12.6 ± 1.2
	Acc <sup>+</sup>	619.4 ± 30.4	0.40 ± 0.08	13.0 ± 0.8
	Klaxon	257.0 ± 22.2	7.42 ± 0.40	13.8 ± 0.9

**Table 4.15:** *In vitro* leaf NADH-nitrate reductase and *in vitro* leaf NADPH-nitrate reductase activities within F<sub>2</sub> populations from STA1010, STA2760, STA4169 and STA3999 and of wild-type barley

*In vitro* NADH-nitrate reductase and *in vitro* NADPH-nitrate reductase activities in the leaf tissue of 7-day-old, green plants, grown in the absence of nitrate, within the F<sub>2</sub> populations from the crosses STA1010 x Klaxon, STA2760 x Klaxon and STA4169 x Golden Promise, within a segregating F<sub>5</sub> population from the cross STA3999 x Tweed and of the wild-type barley cultivars Klaxon, Golden Promise and Tweed. Leaf tissue was extracted with the buffer described by Kuo *et al* (1980) before (nitrate untreated) and after (nitrate treated) treatment with nitrate in the light. Plant growth, leaf nitrite accumulation screens, tissue extraction and enzyme assays were performed as described in Materials and Methods. Column 1 (NADH) shows *in vitro* NADH-nitrate reductase activity, column 2 (NADPH-) shows *in vitro* NADPH-nitrate reductase activity in the absence of pyruvate and LDH and column 3 (NADPH+) shows *in vitro* NADPH-nitrate reductase activity in the presence of pyruvate and LDH.

GP indicates the wild-type cv Golden Promise. All figures are in nmoles nitrite produced/mg protein /h.

Two independent experiments were performed and the data from one of these experiments is shown.

Test	Growth conditions	Plant	NADH	NADPH-	NADPH+
STA1010	Nitrate untreated	Acc <sup>-</sup>	20.1	8.3	0
		Acc <sup>+</sup>	77.4	5.1	0
		Klaxon	15.3	7.4	0
	Nitrate treated	Acc <sup>-</sup>	343.6	58.1	11.9
		Acc <sup>+</sup>	671.4	63.2	15.4
		Klaxon	267.4	72.3	19.9
STA2760	Nitrate untreated	Acc <sup>-</sup>	24.6	10.8	0
		Acc <sup>+</sup>	83.4	15.3	0
		Klaxon	16.3	10.3	0
	Nitrate treated	Acc <sup>-</sup>	368.2	44.1	11.2
		Acc <sup>+</sup>	688.1	67.6	12.7
		Klaxon	356.2	75.7	15.2
STA4169	Nitrate untreated	Acc <sup>-</sup>	27.9	4.2	0
		Acc <sup>+</sup>	65.5	10.4	0
		GP	23.4	5.0	0
	Nitrate treated	Acc <sup>-</sup>	384.4	79.1	16.2
		Acc <sup>+</sup>	540.1	64.2	19.8
		GP	345.3	67.7	8.4
STA3999	Nitrate untreated	Acc <sup>-</sup>	23.1	4.9	0
		Acc <sup>+</sup>	86.2	14.1	0
		Tweed	23.4	5.2	0
	Nitrate treated	Acc <sup>-</sup>	339.2	42.5	11.9
		Acc <sup>+</sup>	692.1	75.8	18.7
		Tweed	298.4	63.8	18.6

#### 4.2.2.5 Northern analysis of STA1010, STA2760, STA4169 and STA3999

Northern analysis as described in Materials and Methods was performed on total RNA from the leaf tissue of the plants described in 4.2.2.4. The partial barley nitrite reductase cDNA clone BNiR1 was used as a probe for nitrite reductase (*nii*) transcript. Barley possesses two nitrate reductase genes, *Nar1* and *Nar7* which encode NADH-nitrate reductase and NAD(P)H-nitrate reductase respectively. The results of NADH-nitrate reductase and NADPH nitrate reductase activity assays (Table 4.15) indicated that NAD(P)H-nitrate reductase activity makes little contribution to the total nitrate reductase activity in the leaf of wild-type plants or *Acc*<sup>+</sup> and *Acc*<sup>-</sup> plants within *nir1* mutant populations and, unlike NADH-nitrate reductase activity, there is little difference in the levels of NAD(P)H activity between these plants. On this basis, nitrate reductase transcript in the leaf was studied using the barley *Nar1* cDNA bNRp30 (Cheng *et al*, 1986) as a probe.

##### 4.2.2.5.1 STA1010

Study of plants within the F<sub>2</sub> population derived from the cross STA1010 × Klaxon and of wild-type cv Klaxon plants (Figure 4.9) demonstrated in both replicate experiments (1010/1 and 1010/2; Fig 4.8) that where plants are grown in the absence of nitrate, leaf *nii* transcript is undetectable in the STA1010 *Acc*<sup>+</sup> plants as well as the *Acc*<sup>-</sup> siblings and wild-type plants (Figure 4.8). Leaf *nar1* transcript of wild-type size (3.5kb) is present in *Acc*<sup>+</sup> plants, and is increased compared to the *Acc*<sup>-</sup> siblings and wild-type plants (Figure 4.8).

After treatment of the plants with nitrate, the two replicate experiments (1010/1 and 1010/2) gave slightly different results. Experiment 1010/1 (Figure 4.8a) shows that STA1010 *Acc*<sup>+</sup> plants possess leaf *nii*



transcript of wild-type size (2.3kb) and at apparently higher levels than *Acc*<sup>-</sup> siblings and wild-type plants. In these samples, leaf *nar1* transcript also appears to be present in higher levels than in *Acc*<sup>-</sup> siblings and wild-type plants (Figure 4.8). However, these apparently increased levels of transcript are most likely due to overloading of the *Acc*<sup>+</sup> RNA sample in the gel (Figure 4.8). In the second replicate experiment, STA1010/2, *Acc*<sup>+</sup> plants also produce leaf *nii* transcript at 2.3kb although there is no leaf *nii* transcript in the *Acc*<sup>-</sup> siblings or in the wild-type after treatment with nitrate (Figure 4.8b), even though methyl viologen nitrite reductase assays have shown normal leaf nitrite reductase activity in these plants (Table 4.11). However, leaf *nar1* transcript is present in similar levels in the STA1010 *Acc*<sup>+</sup> plants, in the *Acc*<sup>-</sup> siblings and in the wild-type plants (Figure 4.8b). This second replicate experiment does, nevertheless, confirm that the homozygous mutant STA1010 synthesises leaf *nii* transcript of wild-type size (2.3kb) and that the homozygous mutant STA1010 also overexpresses leaf *nar1* transcript when grown in the absence of nitrate but possesses wild-type levels of leaf *nar1* transcript when treated with nitrate in the light (Figure 4.8).

#### 4.2.2.5.2 STA2760

Northern analyses of plants within the F<sub>2</sub> population derived from the cross STA2760 × Klaxon and of the wild-type cultivar Klaxon displayed similar results in replicate experiments (2760/1 and 2760/2; Figure 4.9). When grown in the absence of nitrate, the STA2760 *Acc*<sup>+</sup> plants, *Acc*<sup>-</sup> siblings and wild-type plants do not possess detectable leaf *nii* transcript (Figure 4.9). However, the STA2760 *Acc*<sup>+</sup> plants display increased leaf *nar1* transcript levels at 3.5kb as compared to *Acc*<sup>-</sup> sibling and wild-type plants.

When plants were treated with nitrate in the light for 18 hours, leaf *nii* (2.3kb) transcript levels were similar in STA2760 *Acc*<sup>+</sup> plants, *Acc*<sup>-</sup> siblings

and wild-type plants (Figure 4.9). Leaf *nar1* transcript at 3.5kb was also present in levels similar to the *Acc*<sup>-</sup> siblings and wild-type plants (Figure 4.9). Therefore, the homozygous mutant STA2760 synthesises leaf *nii* transcript of wild-type size (2.3kb) and at wild-type levels, and possesses wild-type levels of leaf *nar1* transcript in response to nitrate, although *nar1* transcript appears to be overexpressed in nitrate-untreated plants (Figure 4.9).

#### 4.2.2.5.3 STA4169

Northern analysis of plants within the F<sub>2</sub> population derived from the cross STA4169 × Golden Promise and plants of the wild-type cv Golden Promise showed similar results in two replicate experiments (4169/1 and 4169/2; Figure 4.10). In the absence of nitrate, there was no detectable leaf *nii* transcript or leaf *nar1* transcript in the STA4169 *Acc*<sup>+</sup>, *Acc*<sup>-</sup> sibling or wild-type plants (Figure 4.10).

When treated with nitrate, STA4169 *Acc*<sup>+</sup> plants possessed leaf *nii* transcript of the same size (2.3kb) and at similar levels to the *Acc*<sup>-</sup> sibling and wild-type plants (Figure 4.10). Similarly leaf *nar1* transcript of the wild-type size (3.5kb) and at similar levels to *Acc*<sup>-</sup> sibling and wild-type plants is produced in STA4169 *Acc*<sup>+</sup> plants (Figure 4.10), hence the homozygous mutant STA4169 displays wild-type *nar1* and *nii* transcript regulation in response to nitrate.

Although in one of the replicate experiments (4169/1; Figure 4.10a) the *nii* and *nar1* transcript sizes appeared smaller in the nitrate-treated STA4169 *Acc*<sup>+</sup> plants than in the *Acc*<sup>-</sup> sibling and wild-type plants, the observation that the *Acc*<sup>+</sup> RNA sample appeared retarded in the agarose gel and that both transcripts appeared to differ from the wild-type suggests that this is an artifact of that particular experiment and the other replicate

experiment (4169/2; Figure 4.10b) is more likely to show the true size of the transcripts.

#### 4.2.2.5.4 STA3999

Study of plants within a segregating F<sub>5</sub> population derived from the cross STA3999 x Tweed and plants of the wild-type cv Tweed show in two replicate studies that in the absence of nitrate, the STA3999 Acc<sup>+</sup> plants do not possess detectable *nii* transcript, like the Acc<sup>-</sup> siblings and wild-type plants (figure 4.11). However, STA3999 Acc<sup>+</sup> plants show higher levels of leaf *nar1* transcript at 3.5kb than Acc<sup>-</sup> siblings and wild-type plants (Figure 4.11).

Analysis of plants treated with nitrate demonstrated inconsistencies between the two replicate studies (3999/1 and 3999/2; Figure 4.11). Whilst both experiments show that 3999 Acc<sup>+</sup> plants produce leaf *nii* transcript of wild-type size (2.3kb), experiment 3999/1 (Figure 4.11a) suggests that leaf *nii* transcript is increased in the STA3999 Acc<sup>+</sup> plants as compared to the Acc<sup>-</sup> siblings and wild-type plants, whereas in experiment 3999/2 (Figure 4.11b) this cannot be determined as Acc<sup>-</sup> siblings and wild-type plants did not produce the high levels of leaf *nii* transcript found in 3999/1 (Figure 4.11a) after nitrate treatment. Leaf *nar1* transcript in experiment 3999/1 (Figure 4.11a) was, like *nii* transcript, of wild-type size (3.5kb) and present at levels higher than in the Acc<sup>-</sup> siblings and wild-type (Figure 4.11a) whereas leaf *nar1* transcript in Acc<sup>+</sup> plants from experiment 3999/2 was at approximately wild-type levels after treatment with nitrate (Figure 4.12b). Therefore, the homozygous mutant STA3999 synthesises leaf *nii* and *nar1* transcripts of wild-type size (2.3kb and 3.5kb, respectively) after treatment with nitrate in the light, although it has not been determined whether these are at higher or lower levels than in the wild-type. In addition, the homozygous mutant

STA3999 also produces increased levels of leaf *Nia* transcript in the absence of nitrate as compared to the wild-type.

Although in one of the replicate experiments (3999/2; Figure 4.11b) the *nii* and *nar1* transcript sizes appeared smaller in the nitrate-treated STA3999  $\text{Acc}^+$  plants than in the  $\text{Acc}^-$  sibling and wild-type plants, the observations that the  $\text{Acc}^+$  RNA sample appeared retarded in the agarose gel and that both transcripts appeared to differ from the wild-type suggests that this is an artifact of that particular experiment and the replicate experiment (3999/1; Figure 11a) is more likely to show the true size of the transcripts (Figure 4.11).

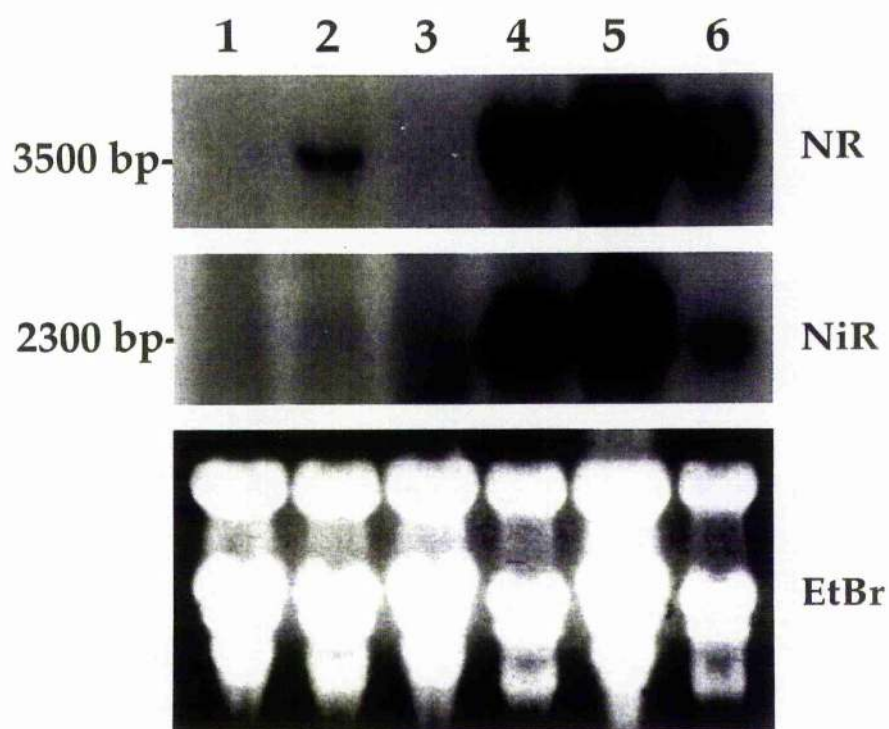
**Figure 4.8:** Steady-state nitrate reductase transcript and nitrite reductase transcript levels within the F<sub>2</sub> population of STA1010 of the wild-type cv Klaxon

Nitrate reductase mRNA (NR) and nitrite reductase mRNA (NiR) levels in leaf tissue of 7-day-old, green plants within the F<sub>2</sub> population from the cross STA1010 × Klaxon and of the wild-type cv Klaxon, the enzyme activities of which are shown in Table 4.11. (a) and (b) correspond to the replicate experiments 1010/1 and 1010/2 respectively. Ethidium bromide-stained agarose gels are also shown (EtBr). Band sizes are shown on the left. Total RNA extraction by the miniprep method and northern analysis, using the partial barley nitrite reductase cDNA BNiR1 (Ward *et al*, 1995) and the barley nitrate reductase (*nar1*) cDNA bNRp30 (Cheng *et al*, 1986) as probes, were performed as described in Materials and Methods.

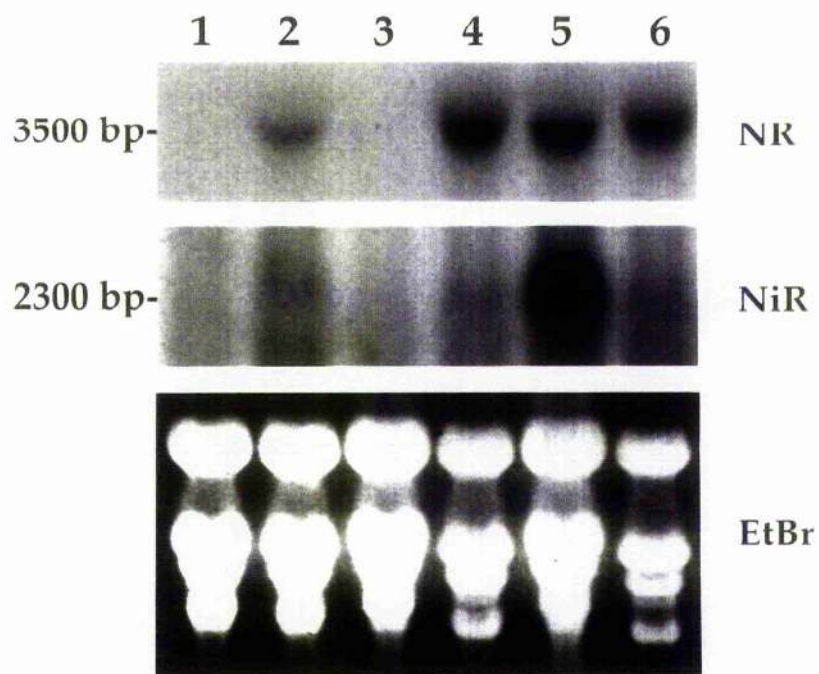
Lanes:

- 1, Nitrate-untreated Acc<sup>-</sup> leaf
- 2, Nitrate-untreated Acc<sup>+</sup> leaf
- 3, Nitrate-untreated wild-type cv Klaxon leaf
- 4, Nitrate-treated Acc<sup>-</sup> leaf
- 5, Nitrate-treated Acc<sup>+</sup> leaf
- 6, Nitrate-treated wild-type cv Klaxon leaf

(a)



(b)





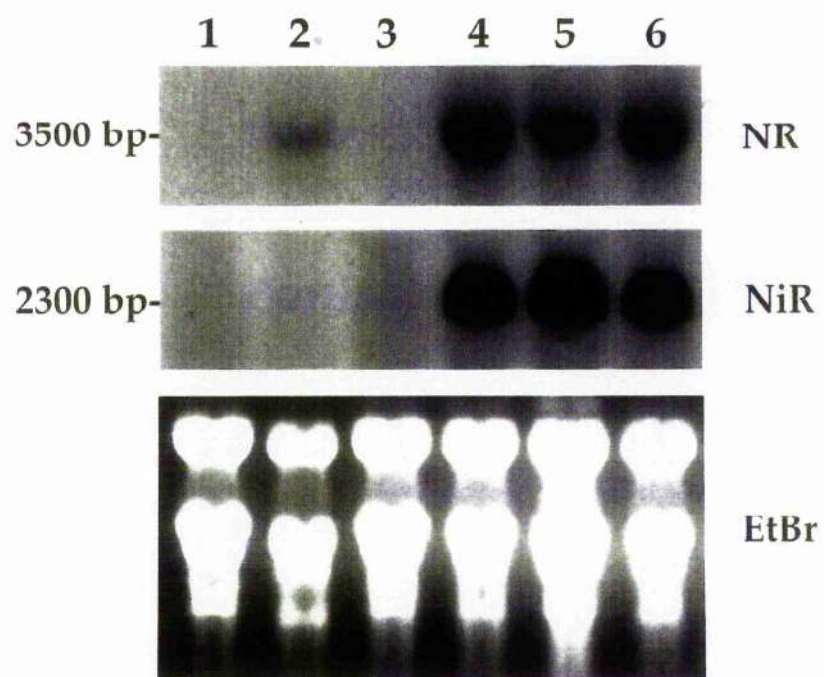
**Figure 4.9:** Steady-state nitrate reductase transcript and nitrite reductase transcript levels within the F<sub>2</sub> population of STA2760 and of the wild-type cv Klaxon

Nitrate reductase mRNA (NR) and nitrite reductase mRNA (NiR) levels in leaf tissue of 7-day-old, green plants within the F<sub>2</sub> population from the cross STA2760 × Klaxon and of the wild-type cv Klaxon, the enzyme activities of which are shown in Table 4.12. (a) and (b) correspond to the replicate experiments 2760/1 and 2760/2 respectively. Ethidium bromide-stained agarose gels are also shown (EtBr). Band sizes are shown on the left. Total RNA extraction by the miniprep method and northern analysis, using the partial barley nitrite reductase cDNA BNiR1 (Ward *et al*, 1995) and the barley nitrate reductase (*nar1*) cDNA bNRp30 (Cheng *et al*, 1986) as probes, were performed as described in Materials and Methods.

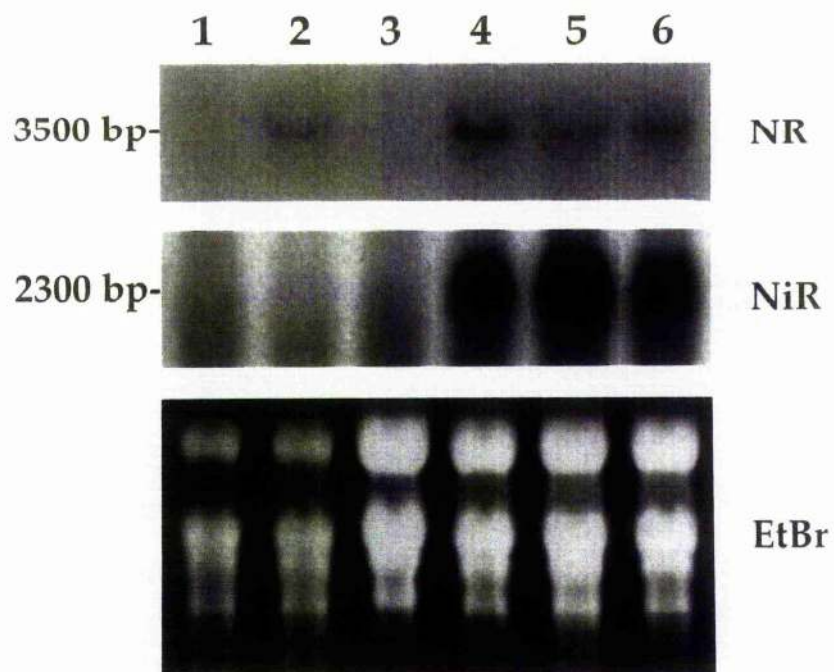
Lanes:

- 1, Nitrate-untreated Acc<sup>-</sup> leaf
- 2, Nitrate-untreated Acc<sup>+</sup> leaf
- 3, Nitrate-untreated wild-type cv Klaxon leaf
- 4, Nitrate-treated Acc<sup>-</sup> leaf
- 5, Nitrate-treated Acc<sup>+</sup> leaf
- 6, Nitrate-treated wild-type cv Klaxon leaf

(a)



(b)





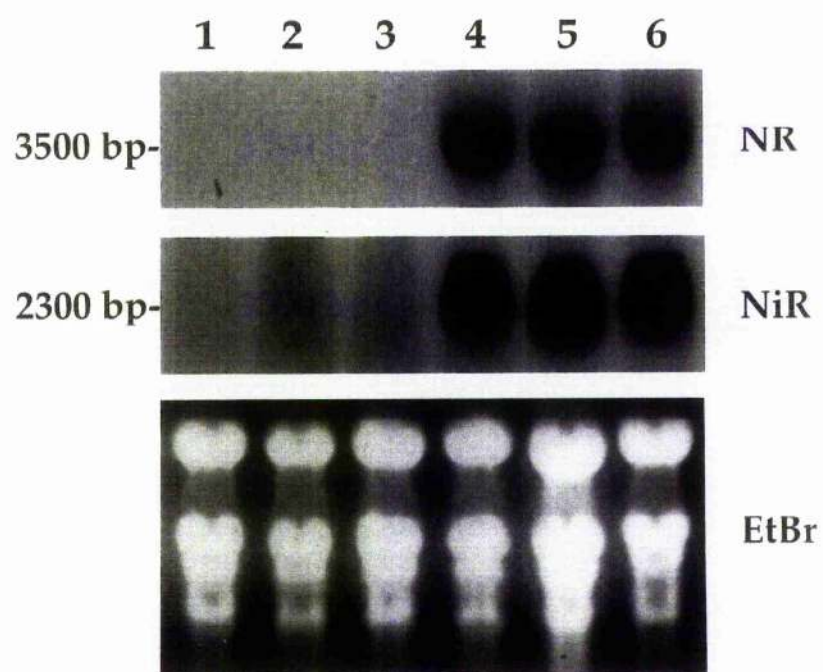
**Figure 4.10:** Steady-state nitrate reductase transcript and nitrite reductase transcript levels within the F<sub>2</sub> population of STA4169 and of the wild-type cv Golden Promise

Nitrate reductase mRNA (NR) and nitrite reductase mRNA (NiR) levels in leaf tissue of 7-day-old, green plants within the F<sub>2</sub> population from the cross STA4169 x Golden Promise and of the wild-type cv Golden Promise, the enzyme activities of which are shown in Table 4.13. (a) and (b) correspond to the replicate experiments 4169/1 and 4169/2 respectively. Ethidium bromide-stained agarose gels are also shown (EtBr). Band sizes are shown on the left. Total RNA extraction by the miniprep method and northern analysis, using the partial barley nitrite reductase cDNA BNiR1 (Ward *et al*, 1995) and the barley nitrate reductase (*nar1*) cDNA bNRp30 (Cheng *et al*, 1986) as probes, were performed as described in Materials and Methods.

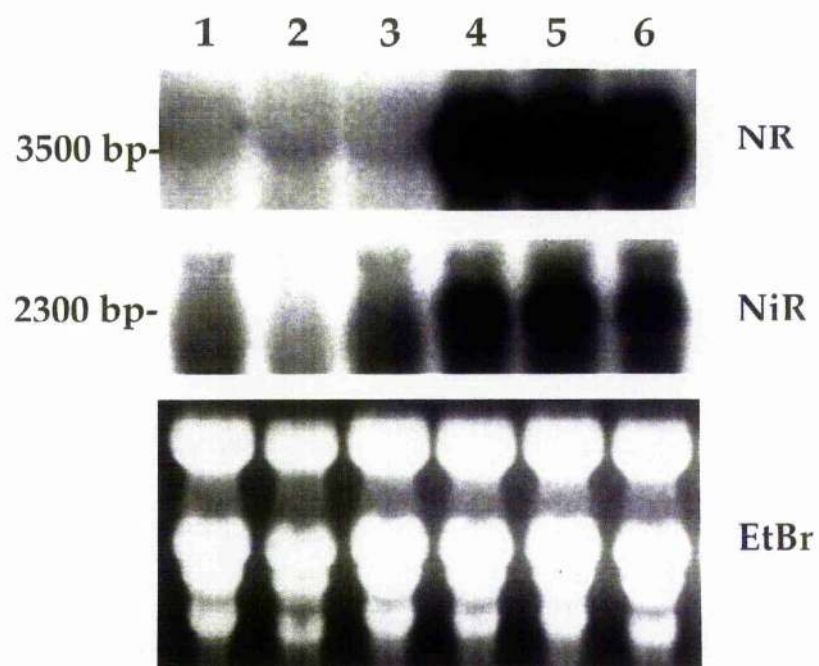
Lanes:

- 1, Nitrate-untreated Acc<sup>-</sup> leaf
- 2, Nitrate-untreated Acc<sup>+</sup> leaf
- 3, Nitrate-untreated wild-type cv Golden Promise leaf
- 4, Nitrate-treated Acc<sup>-</sup> leaf
- 5, Nitrate-treated Acc<sup>+</sup> leaf
- 6, Nitrate-treated wild-type cv Golden Promise leaf

(a)



(b)



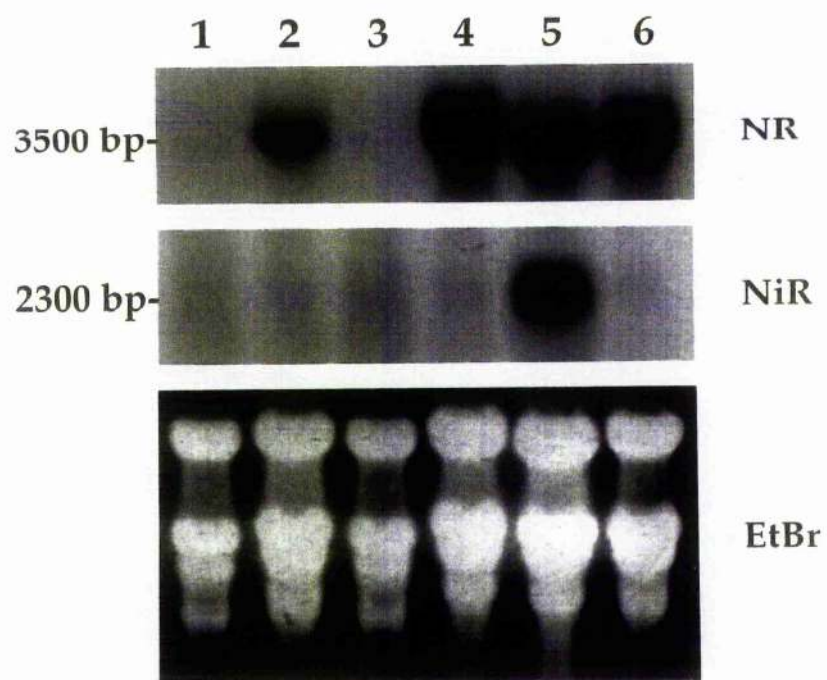
**Figure 4.11: Steady-state nitrate reductase transcript and nitrite reductase transcript levels within a segregating F<sub>5</sub> population of STA3999 of the wild-type cv Tweed**

Nitrate reductase mRNA (NR) and nitrite reductase mRNA (NiR) levels in leaf tissue of 7-day-old, green plants within a segregating F<sub>5</sub> population from the cross STA3999 × Tweed and of the wild-type cv Tweed, the enzyme activities of which are shown in Table 4.14. (a) and (b) correspond to the replicate experiments 3999/1 and 3999/2 respectively. Ethidium bromide-stained agarose gels are also shown (EtBr). Band sizes are shown on the left. Total RNA extraction by the miniprep method and northern analysis, using the partial barley nitrite reductase cDNA BNiR1 (Ward *et al*, 1995) and the barley nitrate reductase (*nar1*) cDNA bNRp30 (Cheng *et al*, 1986) as probes, were performed as described in Materials and Methods.

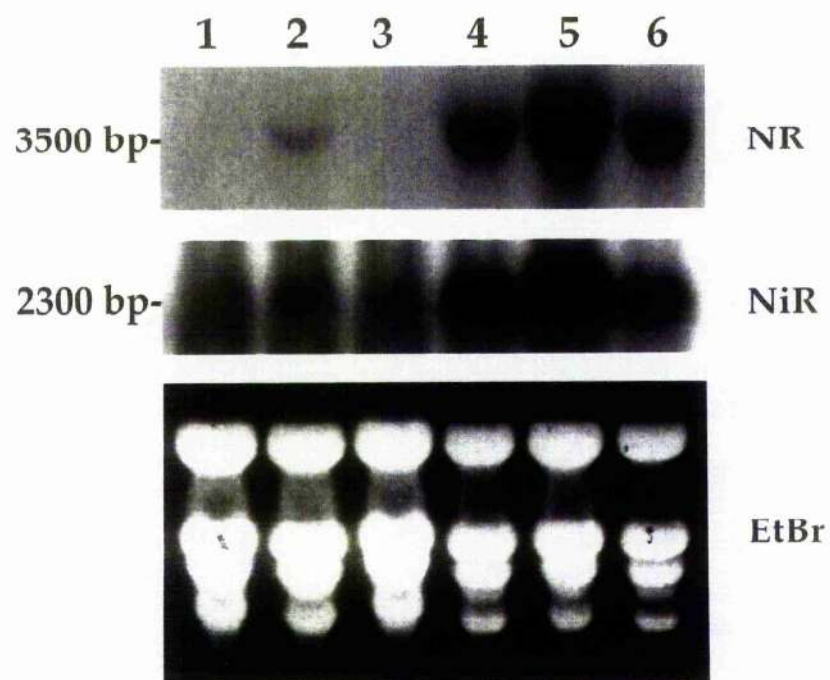
Lanes:

- 1, Nitrate-untreated Acc<sup>-</sup> leaf
- 2, Nitrate-untreated Acc<sup>+</sup> leaf
- 3, Nitrate-untreated wild-type cv Tweed leaf
- 4, Nitrate-treated Acc<sup>-</sup> leaf
- 5, Nitrate-treated Acc<sup>+</sup> leaf
- 6, Nitrate-treated wild-type cv Tweed leaf

(a)



(b)



#### 4.2.2.6 Timecourse of STA3999

To further investigate the regulation of leaf *nii* transcript and leaf *nar1* transcript in STA3999, a timecourse of their induction by nitrate was performed.

Seven-day-old green plants, grown in the absence of nitrate, within a segregating F<sub>5</sub> population derived from the cross STA3999 x Tweed and of the wild-type cv Tweed were treated with 25mM potassium nitrate in the light and leaf tissue samples were taken for enzyme assays, nitrate determination and northern analysis before the addition of nitrate (0 hours) and at 2, 4, 6, 8, 12, 16 and 18 hours after exposure to nitrate.

##### 4.2.2.6.1 *In vitro* leaf enzyme activities

*In vitro* methyl viologen nitrite reductase assays show that STA3999 Acc<sup>+</sup> plants possess a low leaf nitrite reductase activity which is not nitrate inducible (Table 4.16) whereas leaf nitrite reductase activity is induced to high levels by nitrate in the Acc<sup>-</sup> siblings and wild-type plants and an increase in nitrite reductase activity is detectable within two hours of exposure to nitrate (Table 4.16). *In vitro* NADH-nitrate reductase activities show that at all of the sampling times in this experiment, the nitrate reductase activity in the Acc<sup>+</sup> plants is greater than that in the Acc<sup>-</sup> siblings or in the wild-type (Table 4.16). Leaf nitrate content over the duration of the timecourse is similar in STA3999 Acc<sup>+</sup> plants, Acc<sup>-</sup> siblings and wild-type plants (Table 4.16).

**Table 4.16:** *In vitro* enzyme activity and nitrate levels in leaf tissue within a segregating F<sub>5</sub> population from STA3999 and of the wild-type cv Tweed

*In vitro* NADH-nitrate reductase activity (NADH-NR), *in vitro* methyl viologen nitrite reductase activity (MV-NiR) and nitrate content in the leaf tissue of 7-day-old, green plants, grown in the absence of nitrate, within a segregating F<sub>5</sub> population from STA3999 and of the wild-type cv Tweed. Tissue was extracted with the buffer described by Kuo *et al* (1980) after treatment with 25mM potassium nitrate in the light for 0, 2, 4, 6, 8, 12, 16 and 18 hours. Plant growth, leaf nitrite accumulation screens, tissue extraction, enzyme assays and nitrate determinations were performed as described in Materials and Methods. LANE indicates the number of the lane in Figure 4.12 in which the corresponding total RNA sample is loaded.

Two independent experiments were performed and the data from one of these experiments is shown.



Time (hours)	Plant	NADH- NR (nmol nitrite produced/mg protein/h)	MV-NiR ( $\mu$ mol nitrite reduced/mg protein/h)	Nitrate content ( $\mu$ mol/g fresh wt)	LANE
0	Acc <sup>-</sup>	15	0.82	0	1
	Acc <sup>+</sup>	50	0.42	0	2
	Tweed	12	0.94	0	3
2	Acc <sup>-</sup>	40	1.73	1.1	4
	Acc <sup>+</sup>	130	0.40	1.0	5
	Tweed	38	1.86	1.2	6
4	Acc <sup>-</sup>	75	2.44	2.7	7
	Acc <sup>+</sup>	210	0.38	2.6	8
	Tweed	70	2.67	2.6	9
6	Acc <sup>-</sup>	110	3.08	4.4	10
	Acc <sup>+</sup>	290	0.41	3.8	11
	Tweed	103	3.42	4.0	12
8	Acc <sup>-</sup>	150	4.02	6.0	13
	Acc <sup>+</sup>	380	0.32	5.2	14
	Tweed	140	4.24	5.1	15
12	Acc <sup>-</sup>	210	5.57	8.7	16
	Acc <sup>+</sup>	460	0.39	7.9	17
	Tweed	201	5.83	8.2	18
16	Acc <sup>-</sup>	270	7.41	12.1	19
	Acc <sup>+</sup>	550	0.42	10.5	20
	Tweed	265	7.23	10.8	21
18	Acc <sup>-</sup>	300	7.88	13.4	22
	Acc <sup>+</sup>	600	0.35	11.3	23
	Tweed	280	7.31	12.2	24

#### 4.2.2.6.2 Northern analysis

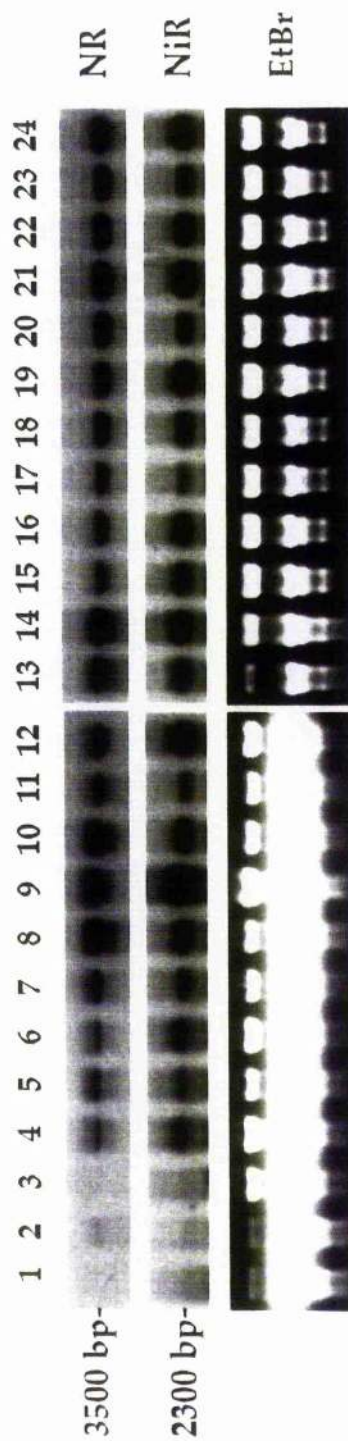
The effect of nitrate on steady-state leaf *nii* and leaf *nar1* transcript level is less clear than the effect of nitrate on nitrite reductase and nitrate reductase activity levels. In order to ensure that conditions remained the same for all RNA samples from this timecourse, the electrophoretic agarose gel was divided into two halves (but not separated) and the total RNA preparations from the 0-6 hour samples were fractionated through the top half of the gel and the total RNA preparations from the 8-18 hour samples through the lower half (Figure 4.12). As the positively-charged ethidium bromide, used to visualise the RNA, was present in the RNA loading buffer, excess ethidium bromide from the lower half of the gel migrated towards the negative electrode at the top of the gel and consequently obscured the visualisation of the 0-6 hour RNA samples and rendering the visual verification of equal RNA loadings difficult (Figure 4.12). However, the largest ribosomal RNA band in the preparations was still visible and was used as a guide to RNA loading. As observed in the northern analysis of STA3999 previously (4.2.2.5.4), leaf *nii* transcript is not detectable in either  $\text{Acc}^+$  plants,  $\text{Acc}^-$  siblings or wild-type plants grown in the absence of nitrate, whereas under the same conditions leaf *nar1* transcript is increased over  $\text{Acc}^-$ /wild-type levels in the  $\text{Acc}^+$  plants (0 hours; Figure 4.12). Leaf *nii* transcript of wild-type size (2.3kb) is detectable in  $\text{Acc}^+$  plants after 2 hours exposure to nitrate and levels at 2 and 4 hours after nitrate-treatment are similar to those in the  $\text{Acc}^-$  sibling and wild-type plants (Figure 4.12). Although there is actually a greater level of leaf *nii* transcript in the wild-type as compared to  $\text{Acc}^+$  after 4 hours exposure to nitrate, this increase can be accounted for by unequal loading of RNA in the gel (Figure 4.12). Leaf *nar1* transcript is present at higher levels in the STA3999  $\text{Acc}^+$  plants than in the  $\text{Acc}^-$  sibling or wild-type plants after 2 and 4 hours exposure to nitrate



(Figure 4.12). However, after 6 hours exposure to nitrate the leaf *nii* transcript level in STA3999  $\text{Acc}^+$  plants has dropped below the levels found in  $\text{Acc}^-$  siblings and wild-type plants and the leaf *nar1* transcript level has also dropped below  $\text{Acc}^-$  sibling and wild-type levels (Figure 4.12). After 8 hours exposure to nitrate, the leaf *nii* transcript level in STA3999  $\text{Acc}^+$  plants increases to  $\text{Acc}^-$  sibling and wild-type levels and leaf *nar1* transcript level also increases in  $\text{Acc}^+$  plants, to greater than  $\text{Acc}^-$  sibling and wild-type levels (Figure 4.12). However, after 12 hours of nitrate treatment leaf *nii* transcript levels in STA3999  $\text{Acc}^+$  have again dropped below  $\text{Acc}^-$  sibling and wild-type levels and leaf *nar1* transcript in STA3999  $\text{Acc}^+$  has decreased to  $\text{Acc}^-$  sibling and wild-type levels and these levels are constant up to and including the final sampling time of 18 hours after nitrate treatment.

Figure 4.12: Steady-state nitrate reductase transcript and nitrite reductase transcript levels in the leaf tissue of a segregating F5 population from STA3999 and of the wild-type cv Tweed

Steady-state nitrate reductase (*nar1*) mRNA (NR) and nitrite reductase (*nir*) mRNA (NiR) levels in the leaf tissue of the F5 STA3999 and wild-type cv Tweed plants described in Table 4.16. Lanes are loaded as described in Table 4.16.



#### 4.2.2.7 Growth characteristics of STA1010, STA2760 and STA4169

When 7-day-old, green plants, grown in the absence of nitrate, within the F<sub>2</sub> populations derived from the crosses STA1010 × Klaxon (Figure 4.13a), STA2760 × Klaxon (Figure 4.14a) and STA4169 × Golden Promise (Figure 4.15a) were transferred to compost in the light, Acc<sup>-</sup> plants within the F<sub>2</sub> populations grew to maturity, flowered and set seed in a similar manner to wild-type plants and whilst Acc<sup>+</sup> plants within the F<sub>2</sub> populations produced new leaves, these began to wither from the tip downwards and subsequently the older leaves withered in the same manner. Withering could be detected within four days of transfer and within 12 days (STA1010 and STA2760; Figure 4.13b and 4.14b, respectively) or 14 days (STA4169; Figure 4.15b) of transfer the plants were dead.

**Figure 4.13:** Growth of individuals within the F<sub>2</sub> population from STA1010 x Klaxon and of the wild-type cv Klaxon

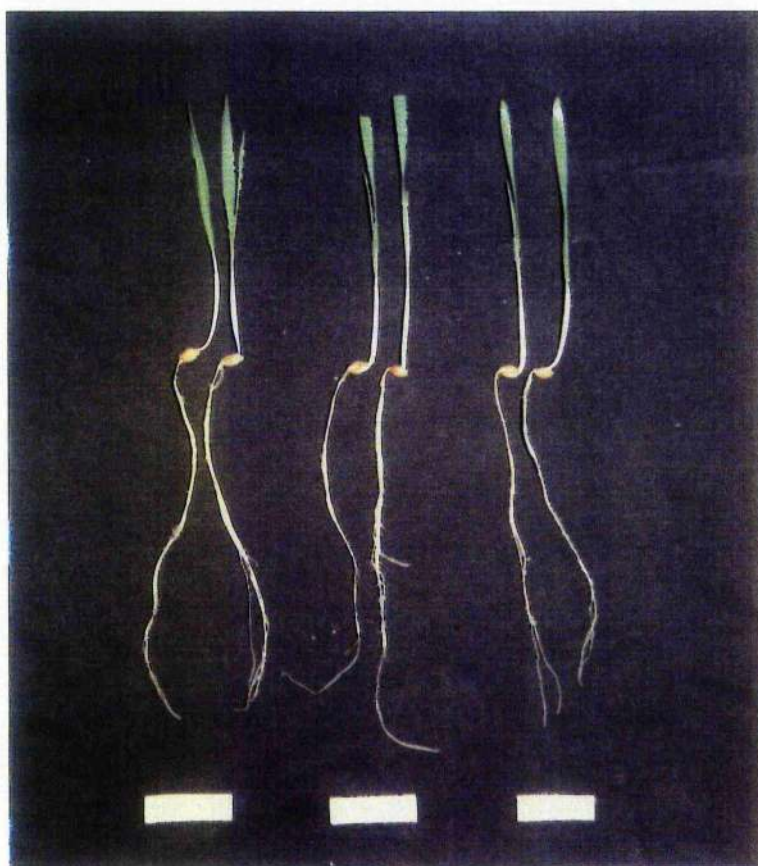
Plants were grown on nitrate-free vermiculite for (a) 7 days and photographed before transfer to compost. Plants were then photographed (b) 12 days after transfer.

(a) F<sub>2</sub> Acc<sup>-</sup> plants are shown on the *left* (average leaf length, 6.2cm); F<sub>2</sub> Acc<sup>+</sup> plants are shown in the *centre* (average leaf length, 6.2cm); plants of the wild-type cv Klaxon are shown on the *right* (average leaf length, 6.2cm).

(b) F<sub>2</sub> Acc<sup>-</sup> plants are shown on the *left* (average leaf length, 31.0cm); F<sub>2</sub> Acc<sup>+</sup> plants are shown in the *centre* (average leaf length, 12.0cm); plants of the wild-type cv Klaxon are shown on the *right* (average leaf length, 30.2cm).

Plant growth and leaf nitrite accumulation screens were performed as described in Materials and Methods.

(a)



(b)



**Figure 4.14:** Growth of individuals within the F<sub>2</sub> population from STA2760 x Klaxon and of the wild-type cv Klaxon

Plants were grown on nitrate-free vermiculite for (a) 7 days and photographed before transfer to compost. Plants were then photographed (b) 12 days after transfer.

(a) F<sub>2</sub> Acc<sup>-</sup> plants are shown on the *left* (average leaf length, 6.3cm); F<sub>2</sub> Acc<sup>+</sup> plants are shown in the *centre* (average leaf length, 6.2cm); plants of the wild-type cv Klaxon are shown on the *right* (average leaf length, 6.5cm).

(b) F<sub>2</sub> Acc<sup>-</sup> plants are shown on the *left* (average leaf length, 33.0cm); F<sub>2</sub> Acc<sup>+</sup> plants are shown in the *centre* (average leaf length, 13.0cm); plants of the wild-type cv Klaxon are shown on the *right* (average leaf length, 30.5cm).

Plant growth and leaf nitrite accumulation screens were performed as described in Materials and Methods.



(a)



(b)





**Figure 4.15:** Growth of individuals within the F<sub>2</sub> population from STA4169 x Golden Promise and of the wild-type cv Golden Promise

Plants were grown on nitrate-free vermiculite for (a) 7 days and photographed before transfer to compost. Plants were then photographed (b) 12 days after transfer.

(a) F<sub>2</sub> Acc<sup>-</sup> plants are shown on the *left* (average leaf length, 6.5cm); F<sub>2</sub> Acc<sup>+</sup> plants are shown in the *centre* (average leaf length, 6.3cm); plants of the wild-type cv Golden Promise are shown on the *right* (average leaf length, 6.8cm).

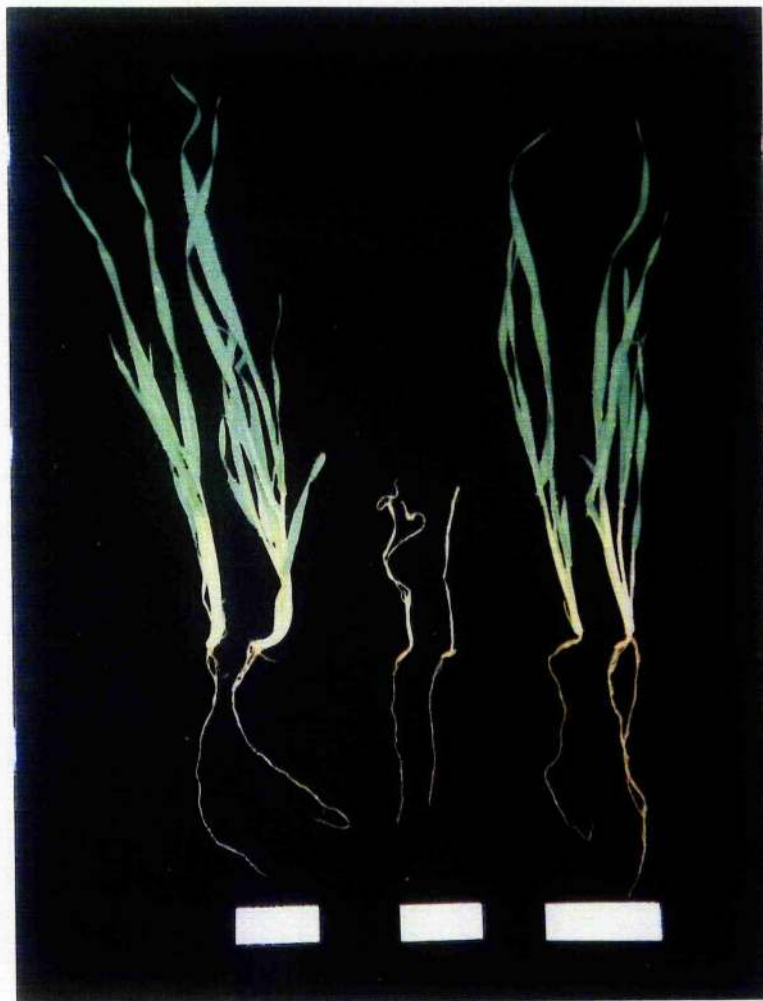
(b) F<sub>2</sub> Acc<sup>-</sup> plants are shown on the *left* (average leaf length, 32.3cm); F<sub>2</sub> Acc<sup>+</sup> plants are shown in the *centre* (average leaf length, 13.1cm); plants of the wild-type cv Golden Promise are shown on the *right* (average leaf length, 28.1cm).

Plant growth and leaf nitrite accumulation screens were performed as described in Materials and Methods.

(a)



(b)



#### 4.2.2.7 *In vivo* leaf nitrite accumulation in STA1010, STA2760 and STA4169

Accumulation of nitrite in the leaves of 7-day-old, green, nitrate-untreated NiR-CRM-minus plants within the F<sub>2</sub> populations derived from the crosses STA1010 x Klaxon, STA2760 x Klaxon and STA4169 x Golden Promise could be detected within 2 hours of exposure to 50mM potassium nitrate in the light (Figure 4.16). Nitrite accumulation in these plants continued over the course of the experiment, and nitrite levels in the STA4169 NiR-CRM-minus plants were lower than those in STA1010 and STA2760 NiR-CRM-minus plants after 18 hours of exposure to nitrate in the light (570nmoles nitrite/g fresh wt, 901nmoles nitrite/g fresh wt and 946nmoles nitrite/g fresh wt respectively; Figure 4.16). NiR-CRM-plus sibling plants and wild-type plants did not accumulate after nitrate treatment (Figure 4.16).

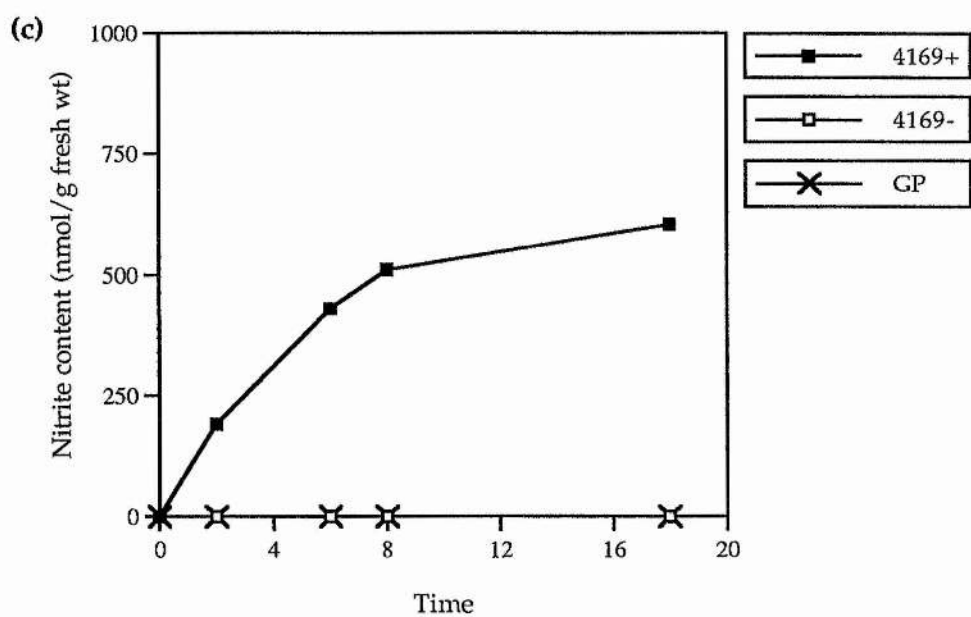
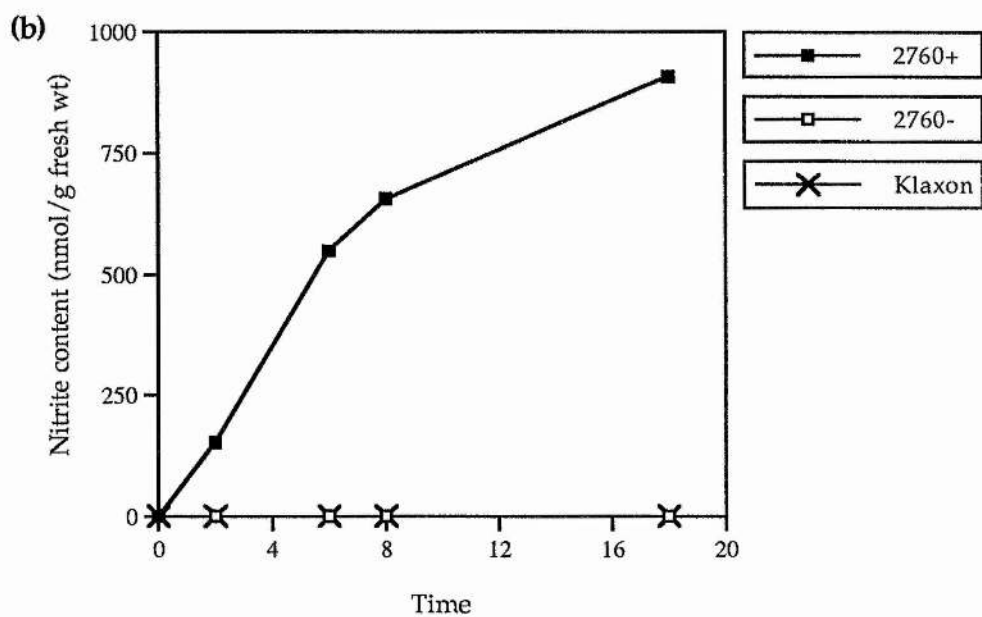
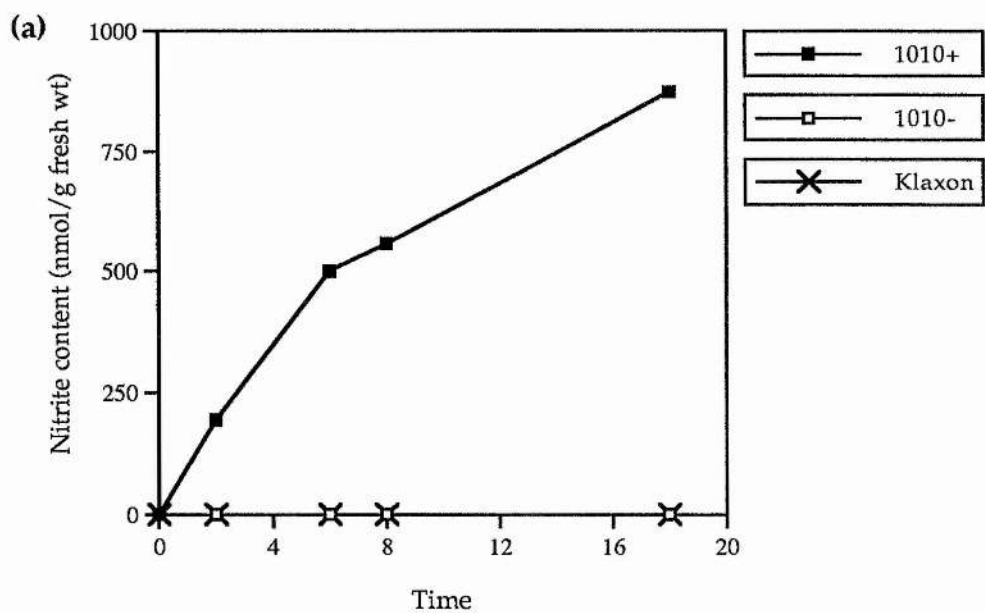
**Figure 4.16:** *In vivo* leaf nitrite accumulation within F<sub>2</sub> populations from STA1010, STA2760 and STA4169 and of wild-type barley

Seven-day-old, green plants, grown in the absence of nitrate, were treated with 50mM potassium nitrate in the light at 0 hours and leaf nitrite concentrations were determined at 0, 2, 6, 8 and 18 hours. Plant growth and leaf nitrite determinations were performed as described in Materials and Methods. Two independent experiments were performed in duplicate and the data from one of these experiments is shown.

(a) Plants within the F<sub>2</sub> population from the cross STA1010 x Klaxon and of the wild-type cv Klaxon. 1010- indicates plants within the F<sub>2</sub> population which retain nitrite reductase cross-reacting material (NiR-CRM); 1010+ indicates plants within the F<sub>2</sub> population which lack detectable NiR-CRM.

(b) Plants within the F<sub>2</sub> population from the cross STA2760 x Klaxon and of the wild-type cv Klaxon. 2760- indicates plants within the F<sub>2</sub> population which retain NiR-CRM; 2760+ indicates plants within the F<sub>2</sub> population which lack detectable NiR-CRM.

(c) Plants within the F<sub>2</sub> population from the cross STA4169 x Golden Promise and from the wild-type cv Golden Promise. 4169- indicates plants within the F<sub>2</sub> population which retain NiR-CRM; 4169+ indicates plants within the F<sub>2</sub> population which lack detectable NiR-CRM; GP denotes the wild-type cv Golden Promise.



## 4.3 DISCUSSION

### 4.3.1 Isolation of F<sub>2</sub> populations from STA1010, STA2760 and STA4169

Cross-pollinations were successfully performed by W.T.B. Thomas, SCRI, Invergowrie, UK, between the three homozygous mutants STA1010, STA2760 and STA4169 and the wild-type cultivars from which they were isolated (Klaxon, Klaxon and Golden Promise respectively), yielding totals of 85, 63 and 64 F<sub>1</sub> seed respectively (Table 4.9), of which 55, 42 and 42 seed respectively were cultivated and allowed to self-pollinate to produce a large population of F<sub>2</sub> seed. Previous attempts at cross-pollinations had been unsuccessful, mainly due to difficulty in maintaining selections in hydroponic culture and synchronising the growth of these selections with the compost-grown wild-type cultivars (Duncanson, 1990). This problem has been overcome by cultivating several wild-type individuals at different times to provide a range of wild-type plants at different developmental stages.

Studies performed on the *nir1* mutants suggested there is little or no phenotypic difference between the M and F populations for each mutant, and characterisation of the F<sub>2</sub> populations derived from the crosses STA1010 x Klaxon, STA2760 x Klaxon and STA4169 x Golden Promise as well as the segregating F<sub>5</sub> populations derived from the cross STA3999 x Tweed (Duncanson *et al*, 1993) are discussed below.

### 4.3.2 Genetics of defects in STA1010, STA2760 and STA4169

The leaf nitrite-accumulating phenotype was inherited within the F<sub>2</sub> populations derived from STA1010 x Klaxon, STA2760 x Klaxon and STA4169 x Golden Promise in ratios not significantly different at the 5% level to the Mendelian ratio of 1:3 nitrite accumulators:nitrite non-accumulators



(Table 4.10), and it was concluded that the leaf nitrite-accumulating phenotype was due to a recessive mutation in a single nuclear gene.

Crosses between heterozygous STA1010 and STA4169 M individuals and between heterozygous STA2760 and STA4169 M individuals (Table 4.7) produced F<sub>1</sub> seed which segregated in ratios not significantly different from the Mendelian 1:3 ratio of nitrite-accumulators:nitrite non-accumulators (Table 4.8). It was concluded from these data that STA1010 and STA4169, STA2760 and STA4196, and therefore STA1010 and STA2760 were allelic for the defective locus. Other studies by J.L.Wray (unpublished) have shown that the mutations in the selections STA3999 and STA1010 are allelic, therefore the four selections characterised here are defective at the *Nir1* locus.

#### 4.3.3 Biochemical characterisation of *nir1* mutants

Immunoblot analysis of nitrite reductase cross-reacting material has demonstrated that the three homozygous mutants STA1010, STA2760 and STA4169 lack detectable nitrite reductase cross-reacting material in the leaf and root (Figure 4.8), as does the homozygous mutant STA3999 (Duncanson *et al*, 1993). It is concluded that the *Nir1* locus is responsible for the production of nitrite reductase protein in both the leaf and root of barley. This being the case, further studies were conducted only in leaf tissue due to the relatively small amounts of tissue required for analysis as compared to root.

Two independent *in vitro* enzyme assay experiments were performed under the same conditions for each of the four *nir1* mutants using leaf tissue from plants within F populations and there was little variation between the replicated experiments for each *nir1* mutant. *In vitro* methyl viologen nitrite reductase activity assays show that the four *nir1* mutants possess only 50% of the wild-type level of leaf nitrite reductase activity when grown in the

absence of nitrate and only 5-8% of the wild-type levels of leaf nitrite reductase activity after treatment with nitrate, and this low activity in the *nir1* mutants is not nitrate inducible (Tables 4.11-4.13). This suggests that the *Nir1* locus is responsible for both the basal level of nitrite reductase activity found in plants not treated with nitrate, as well as the inducible levels of nitrite reductase activity found in plants treated with nitrate. Studies by Duncanson *et al* (1993) suggest that *in vitro* methyl viologen nitrite reductase activity in the homozygous *nir1* mutant STA3999 is approximately 10% of the level in wild-type plants, but use of a different tissue extraction buffer here (Kuo *et al*, 1980) gives a value slightly closer to the expected 2%, suggested by immunoprecipitation of nitrite reductase cross-reacting material from STA3999 (Duncanson *et al*, 1993).

*In vitro* NADH-nitrate reductase activity assays on plants grown in the absence of nitrate show an approximate fourfold increase in nitrate reductase activity in the leaf of the three homozygous *nir1* mutants STA1010, STA2760 and STA3999 over wild-type levels (Tables 4.11-4.12, 4.14). However, only a twofold increase in nitrate reductase activity over wild-type levels was observed in the homozygous *nir1* mutant STA4169 (Table 4.13). *In vitro* NADH-nitrate reductase activity assays performed on plants treated with nitrate in the light show a 2-2.5 times increase in leaf nitrate reductase activity in the homozygous *nir1* mutants STA1010, STA2760 and STA4169 over wild-type levels (Tables 4.11-4.12, 4.14). However, the homozygous *nir1* mutant STA4169 displayed only a 1.5-2 times increase in leaf NADH-nitrate reductase activity over wild-type levels under the same conditions (Table 4.13). This suggests a difference in regulation of nitrate reductase between the selections STA1010, STA2760 and STA3999 and the selection STA4169. In all cases *Acc*<sup>-</sup> siblings and the wild-type plants displayed similar enzyme activities to each other (Tables 4.11-4.14). *In vitro* NADPH-nitrate reductase activities in the *nir1* mutants show that NAD(P)H-nitrate activity is similar to



wild-type levels, hence the higher levels of nitrate reductase activity found in homozygous *nir1* mutants is due to NADH-nitrate reductase, hence to *Nar1* gene expression rather than *Nar7* gene expression and *Nar7* gene expression is not increased as it is in *Nar1* mutants (Dailey *et al*, 1982b).

Northern analyses of leaf tissue samples from the experiments described above showed that the homozygous *nir1* mutants STA1010 and STA2760 produce nitrite reductase (*nii*) transcript of wild-type size (2.3kb) and at approximately wild-type levels in the leaf of plants treated with nitrate in the light (Figures 4.8-4.9) and, as for *Acc*<sup>-</sup> siblings and wild-type plants, leaf *nii* transcript was undetectable in plants grown in the absence of nitrate (Figures 4.9-4.10). Leaf NADH-nitrate reductase (*nar1*) transcript levels in homozygous mutant STA1010 and STA2760 plants grown in the absence of nitrate were greater than wild-type levels, although leaf *nar1* transcript levels in homozygous mutant STA1010 and STA2760 plants treated with nitrate in the light were approximately the same as wild-type levels under the same conditions (Tables 4.8-4.9).

Northern analysis of the homozygous *nir1* mutant STA4169, however, showed differences to STA1010 and STA2760 (Figure 4.10). Leaf *nii* transcript in homozygous mutant STA4169 plants was undetectable in the absence of nitrate, as was leaf *nii* transcript in the *Acc*<sup>-</sup> siblings and wild-type plants, and was of wild-type size (2.3kb) and at wild-type levels in plants treated with nitrate (Figure 4.10), similar to STA1010 and STA2760. Leaf *nar1* transcript, however, was undetectable in the homozygous mutant STA4169 in the absence of nitrate, unlike in the homozygous mutants STA1010 and STA2760, and was present in wild-type levels in the presence of nitrate (Figure 4.10). The lack of overexpression of *nar1* transcript in nitrate-untreated plants may explain the lower NADH-nitrate reductase activities seen in the homozygous mutant STA4169 as compared with STA1010, STA2760 and STA3999.

Replicate northern analyses using the selection STA3999 (Figure 4.11) were unsatisfactory in characterising the regulation of leaf *nii* and leaf *nar1* transcript levels in this selection, although they did confirm that the homozygous mutant STA3999 produces leaf *nii* and *nar1* transcript of wild-type size (2.3kb and 3.5kb, respectively) after treatment with nitrate in the light and that STA3999 overexpresses leaf *nar1* transcript in the absence of nitrate (Figure 4.11). To further investigate the regulation nitrite reductase and nitrate reductase activity levels and transcript in the leaf of STA3999 after treatment with nitrate, a timecourse of exposure to nitrate was performed (Table 4.16; Figure 4.12). The results of this timecourse suggest that up to 8 hours after treatment with nitrate, leaf *nii* transcript is present at wild-type levels and leaf *nar1* transcript is present at above wild-type levels in the homozygous STA3999 mutant (Figure 4.12). However, after 12 hours of nitrate treatment, leaf *nii* transcript synthesis had dropped below wild-type levels and *nar1* transcript presence had dropped to wild-type levels in the mutant (Figure 4.12) where they remained up to 18 hours after treatment with nitrate in the light. There also appeared to be a greater differential between leaf *nii* transcript level and leaf *nar1* transcript level in the homozygous mutant STA3999 than in the *Acc*-siblings wild-type plants which may be of importance in the co-regulation of nitrite reductase and nitrate reductase in *nir1* mutants.

To summarise, biochemical studies using the four *nir1* mutant selections STA1010, STA2760, STA4169 and STA3999 have shown that three of these mutants, STA1010, STA2760 and STA3999, have very similar, if not identical phenotypes. The phenotype of the fourth selection, STA4169, differs from these at both the transcriptional and enzyme activity level.

#### 4.3.4 Growth of *nir1* mutants

*In vivo* leaf nitrite accumulation studies (Figure 4.16) show that in the homozygous *nir1* mutants, nitrite is accumulated within 2 hours of exposure to nitrate in the light and continues to accumulate over an 18 hour period, although nitrite does not accumulate to the same extent in the homozygous mutant STA4169 as in the other selections (Figure 4.16). *In vivo* leaf nitrite accumulation to toxic levels would explain why the homozygous *nir1* mutants only survive up to 12 days (STA1010, STA2760 and STA3999) or 14 days (STA4169) after transfer to compost (Figures 4.13-4.15). The slightly longer survival time of STA4169 as compared to the other selections provides further evidence that the regulation of nitrate reductase in the homozygous *nir1* mutant STA4169 is different to that of the three other *nir1* mutants as lower nitrate reductase activity means a lower accumulation of toxic nitrite and thus longer survival times in a nitrate-rich environment.

#### 4.3.5 Possible identity of the *Nir1* locus

Two main possibilities present themselves with respect to the identity of the *Nir1* locus. The most likely is that it encodes the nitrite reductase apoprotein gene, a mutation in which may be expected to produce the *nir1* phenotype. An alternate possibility is that the locus encodes a regulatory locus which affects nitrite reductase but not nitrate reductase. This would probably encode a protein component of the signal transduction pathway, which mediates nitrate, light and plastidic factor regulation of the nitrite reductase apoprotein gene. If true, this would suggest that the *nir1* mutation inactivates this component and does not allow transcription of the nitrite reductase apoprotein gene. However, as these studies have shown, the *nir1* mutants produce *nii* transcript of wild-type size and at approximately wild-

type levels so it is most unlikely that the *Nir1* locus represents a regulatory component of the nitrate assimilation pathway.

## CHAPTER 5

### RFLP Mapping of the *Nir1* Locus in Barley

## 5.1 INTRODUCTION

The four whole-plant barley mutants STA1010 and STA2760 from cv Klaxon, STA4169 from cv Golden Promise and STA3999 from cv Tweed are defective in nitrite reduction and have been partially characterised (Chapter 4; Duncanson *et al*, 1993) and all possess a recessive mutation in a single nuclear locus, *Nir1*. Mutation at the *Nir1* locus leads to loss of detectable nitrite reductase protein in both leaf and root and to loss of both the "basal" level of nitrite reductase activity, found in plants not treated with nitrate, as well as the "inducible" nitrite reductase activity in leaf and root of plants treated with nitrate in the light. Whilst it is possible that *Nir1* represents a regulatory locus whose product is required for the synthesis of nitrite reductase, but not nitrate reductase, the observation that all four *nir1* mutants possess nitrite reductase (*nii*) transcript of wild-type size and at approximately wild-type levels, suggest that the *Nir1* locus represents the nitrite reductase apoprotein gene, *Nii*. To test this hypothesis, RFLP mapping techniques have been employed to estimate the map distance between the *Nir1* locus and the *Nii* gene.

Genetic analysis of plant genomes has been revolutionised in recent years with the use of molecular genetic tools, including RFLP mapping, for rapidly developing a large number of genetic markers. In the RFLP approach, cloned DNA sequences are used to probe specific regions of a genome for the presence of variations at the DNA sequence level. These variations are detected by restriction endonucleases and revealed by separating DNA fragments according to size by electrophoresis. Polymorphism is seen as differences in the length of genomic DNA fragments homologous to a radiolabelled cloned DNA sequence. Such variation has been termed Restriction Fragment Length Polymorphism (RFLP) (Landry, 1993). RFLP markers were first used as a tool for genetic

analysis when a temperature-sensitive mutation of adenovirus was associated with a specific RFLP (Grodzicker *et al*, 1974). The first use of RFLP as a genetic marker was in mapping genes associated with disease in humans (Reviewed in Landry, 1993) although since then RFLP analysis has been performed for many organisms to construct detailed genetic linkage maps, including barley (Graner *et al*, 1991), maize (Hoisington and Lander, 1987), bean (Nodari *et al*, 1993) and tomato (Vanooijen *et al*, 1994). In fact, recent studies have demonstrated an RFLP between the wheat cultivar Chinese Spring and the barley cultivar Betzes when *Hind*III-digested DNA from the two cultivars was probed with radiolabelled insert from the barley partial nitrite reductase cDNA clone pBNiR1 (Ward *et al*, 1995; J.L. Wray, unpublished). It was subsequently possible to use wheat/barley ditelosomic addition lines (Islam 1983) to assign the *Nii* gene to the long arm of chromosome 6 (J.L. Wray, unpublished).

The map distance between two loci is determined by the recombination frequency after meiosis, which can range from 0-50%. The closer the two loci, the less chance there is of crossover between them and consequently the recombination frequency is lower. Recombination frequency can be measured between a restriction marker (RFLP) and a visible phenotypic marker, thus a genetic map can include both phenotypic and genotypic markers. Therefore the genetic map distance between the *Nir1* locus, a visible phenotypic marker, and the *Nii* gene could be estimated from recombination between the *Nir1* phenotype and a *Nii* RFLP.

In this chapter, RFLP data are presented which indicate that the *Nir1* locus is very tightly linked to, and probably within, the nitrite reductase apoprotein gene, *Nii*.



## 5.2 RESULTS

### 5.2.1 Identification of an RFLP between barley cv Tweed and barley cv Golden Promise

Southern analysis of barley genomic DNA, digested with a range of restriction endonucleases (J.L. Wray, unpublished) demonstrated that when *Dra*I-digested genomic DNA was probed with radiolabelled insert from the partial barley nitrite reductase cDNA clone pBNiR1 (Ward *et al*, 1995) an RFLP is present between the barley wild-type cv Tweed, in which the *nir1* mutant STA3999 was isolated, and the barley wild-type cv Golden Promise (Figure 5.1). The wild-type cv Tweed exhibits a major hybridising band at 11.5kb whilst the wild-type cv Golden Promise exhibits a major hybridising band at 7.5kb. An RFLP was not detected between the wild-type cv Tweed and the Tweed *nir1* mutant STA3999 (Figure 5.1). F<sub>1</sub> populations heterozygous for both the RFLP and the *nir1* mutation (leaf nitrite accumulation/lack of detectable NiR-CRM) were produced by crossing the wild-type cv Golden Promise (RFLP band at 7.5kb) and the homozygous cv Tweed *nir1* mutant STA3999 (RFLP band at 11.5kb) and cosegregation of the mutant phenotype with the Tweed RFLP band, recombination between the mutant phenotype and the Golden Promise RFLP band at 7.5kb and recombination between the wild-type phenotype and the Tweed RFLP band at 11.5kb was looked for in the F<sub>2</sub> population to estimate the recombination frequency. If the *Nir1* locus is the nitrite reductase apoprotein gene *Nii*, then there will be no recombination between the parental phenotypes.

### 5.2.2 Southern Analysis of F<sub>1</sub> Plants from the cross Golden Promise x STA3999

Cross-pollination between the wild-type cv Golden Promise and the homozygous cv Tweed *nir1* mutant STA3999 was performed by W.T.B. Thomas at SCRI, Invergowrie, UK, using cv Golden Promise as the female (pollen recipient) parent. Independent crosses produced four putative F<sub>1</sub> plants, designated F<sub>1</sub>1, F<sub>1</sub>2, F<sub>1</sub>3 and F<sub>1</sub>4. When *Dra*I-digested genomic DNA from these four F<sub>1</sub> plants was probed with BNiR1, two of the plants, F<sub>1</sub>1 and F<sub>1</sub>4, were shown to be heterozygous for the RFLP (Figure 5.1a and 5.1d), whereas the remaining two plants, F<sub>1</sub>2 and F<sub>1</sub>3, were shown to be homozygous for the Golden Promise RFLP band at 7.5kb (Figure 5.1b and 5.1c). This observation suggests that plants F<sub>1</sub>2 and F<sub>1</sub>3 are the result of self-pollination of the cv Golden Promise female parent.

The four F<sub>1</sub> plants were allowed to self-pollinate to produce F<sub>2</sub> seed and these F<sub>2</sub> populations were then examined for co-segregation of the mutant phenotype and the Tweed RFLP band at 11.5kb.

**Figure 5.1: RFLP analysis of F<sub>1</sub> plants derived from the cross Golden Promise x STA3999**

RFLP analysis of *Dra*I-digested DNA probed with radiolabelled insert from the partial barley nitrite reductase cDNA clone pBNiR1 (Ward *et al*, 1995).

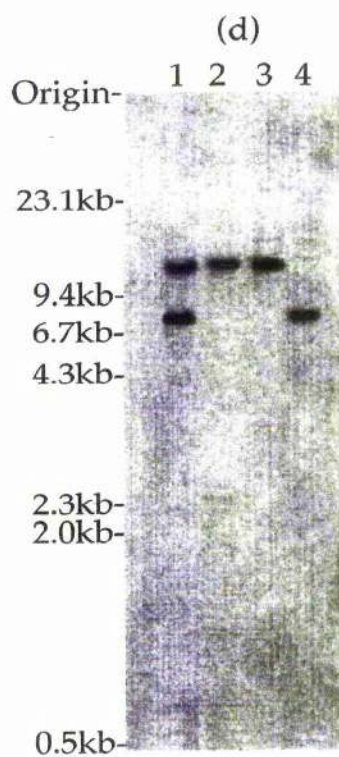
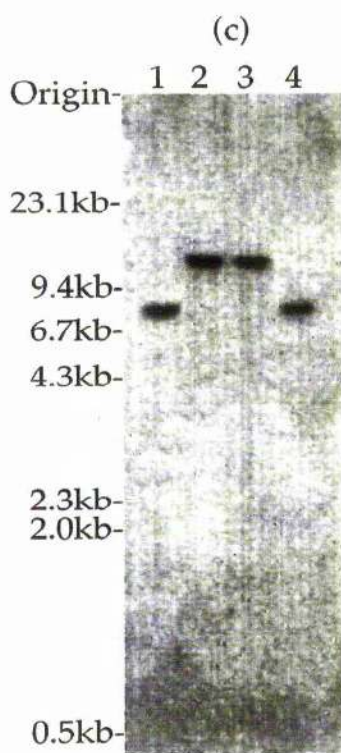
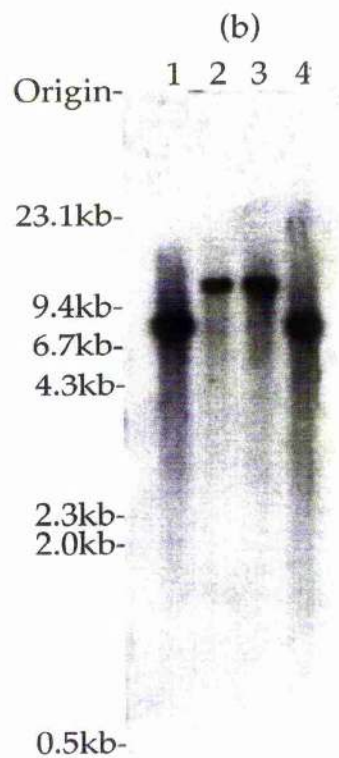
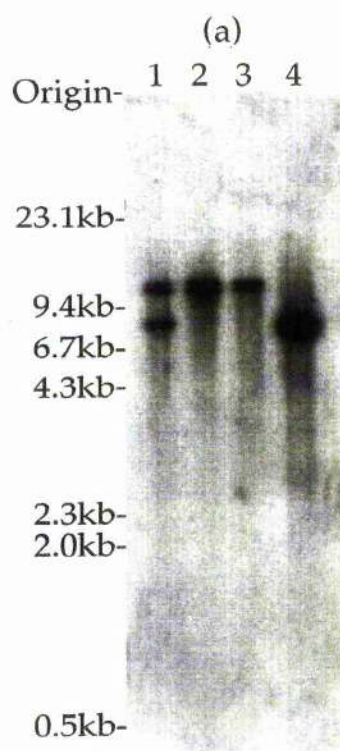
(a) Lane 1, DNA from F<sub>1</sub>1, a putative F<sub>1</sub> plant derived from the cross between the wild-type cv Golden Promise and the cv Tweed *nir1* mutant STA3999; lane 2, DNA from cv Tweed *nir1* mutant STA3999; lane 3, DNA from wild-type cv Tweed; lane 4, DNA from wild-type cv Golden Promise.

(b) As (a) except lane 1, DNA from F<sub>1</sub>2, a putative F<sub>1</sub> plant derived from the cross between the wild-type cv Golden Promise and the cv Tweed *nir1* mutant STA3999.

(c) As (a) except lane 1, DNA from F<sub>1</sub>3, a putative F<sub>1</sub> plant derived from the cross between the wild-type cv Golden Promise and the cv Tweed *nir1* mutant STA3999.

(d) As (a) except lane 1, DNA from F<sub>1</sub>4, a putative F<sub>1</sub> plant derived from the cross between the wild-type cv Golden Promise and the cv Tweed *nir1* mutant STA3999.

DNA extractions and Southern hybridisation were performed as described in Materials and Methods. Marker sizes are shown on the left.



### 5.2.3 Analysis of F<sub>2</sub> Populations from the cross Golden Promise x STA3999

#### 5.2.3.1 Segregation analysis of F<sub>2</sub> individuals

Self-pollination of the F<sub>1</sub> plants F<sub>1</sub>1, F<sub>1</sub>2, F<sub>1</sub>3 and F<sub>1</sub>4 produced 148 F<sub>2</sub>1, 112 F<sub>2</sub>2, 177 F<sub>2</sub>3 and 196 F<sub>2</sub>4 seed. Fourteen individuals from each of these F<sub>2</sub> populations were sown, although only 9, 13, 11 and 8 seed respectively germinated (Table 5.1). Screening for leaf nitrite accumulation as described in Materials and Methods of these F<sub>2</sub> plants demonstrated segregation for leaf nitrite accumulation within the F<sub>2</sub> populations derived from F<sub>1</sub>1 and F<sub>1</sub>4, whereas the F<sub>2</sub> populations derived from F<sub>1</sub>2 and F<sub>1</sub>3 did not segregate for leaf nitrite accumulation (Table 5.1). It was concluded that the putative F<sub>1</sub> plants 2 and 3 were the result of self-pollination of the female Golden Promise parent and these plants were not studied further.

After identification of the nitrite accumulators, the remaining leaf tissue of the seventeen F<sub>2</sub>1 and F<sub>2</sub>4 plants was harvested and *Dra*I-digested DNA from these samples, probed with BNiR1, showed that all F<sub>2</sub> individuals which accumulated nitrite in the leaf (Acc<sup>+</sup>) were also homozygous for the Tweed RFLP band at 11.5kb (Figure 5.2). Those F<sub>2</sub> individuals from the same populations which did not accumulate nitrite in the leaf (Acc<sup>-</sup>) were all either homozygous for the Golden Promise RFLP band at 7.5kb or heterozygous for the RFLP bands (Figure 5.2).

**Table 5.1:** Inheritance of leaf nitrite accumulation within the F<sub>2</sub> populations derived from the cross Golden Promise x STA3999

Population	No. nitrite non-accumulators	No. nitrite accumulators	$\chi^2$ (3:1)
F <sub>21</sub>	5	4	3.51
F <sub>22</sub>	12	0	-
F <sub>23</sub>	11	0	-
F <sub>24</sub>	5	4	0.67

Inheritance of leaf nitrite accumulation within the F<sub>2</sub> populations derived from each of the putative F<sub>1</sub> plants F<sub>11</sub>, F<sub>12</sub>, F<sub>13</sub> and F<sub>14</sub>. Leaf nitrite accumulation screens were performed as described in Materials and Methods. A  $\chi^2$  value of below 3.84 indicates that the segregation ratio is not significantly different at the 5% level from the Mendelian 3:1 ratio.

**Figure 5.2:** Cosegregation of leaf nitrite accumulation and the Tweed RFLP band

Cosegregation of leaf nitrite accumulation with the Tweed RFLP band at 11.5kb in F<sub>2</sub> individuals from the cross Golden Promise x STA3999.

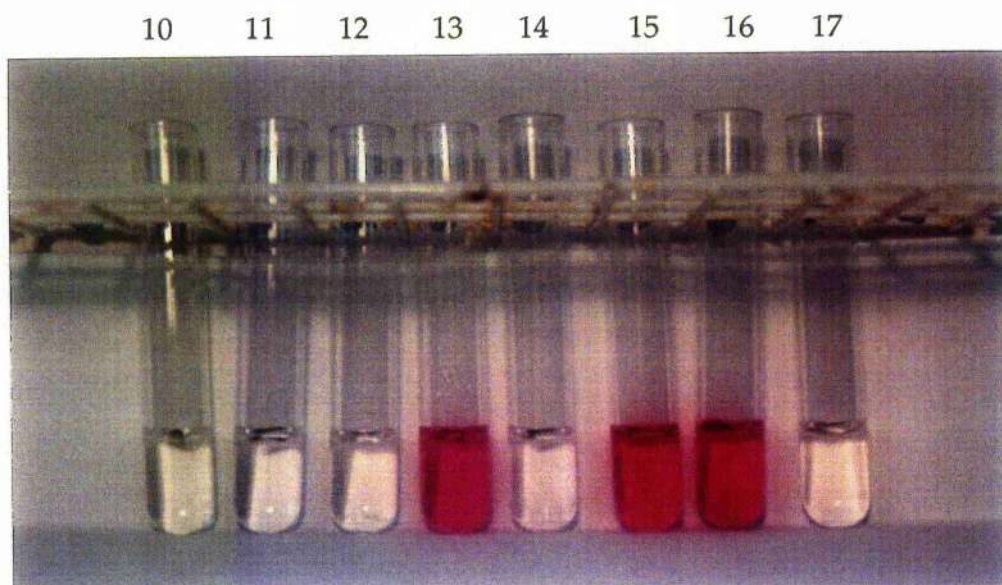
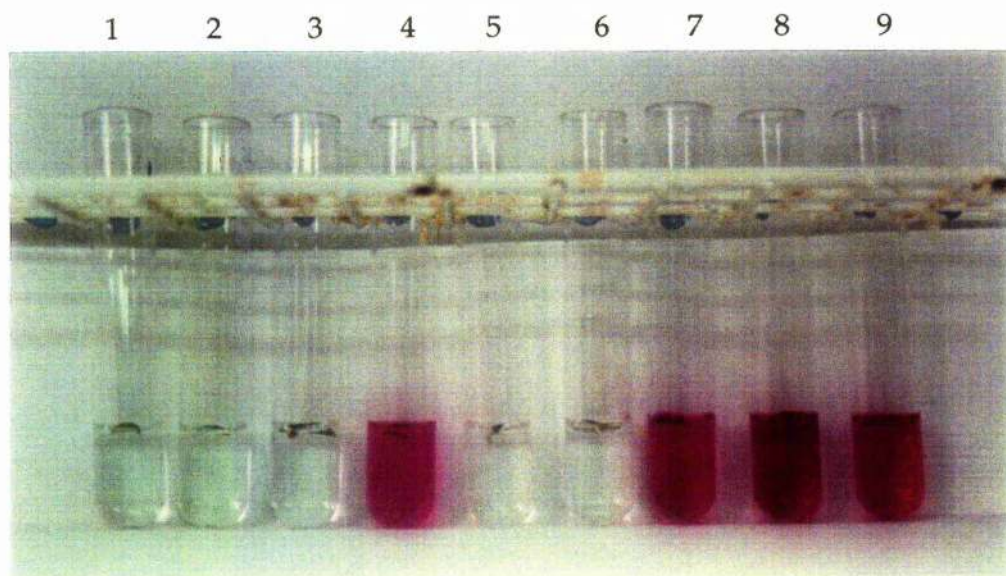
(a) Leaf nitrite accumulation in individual plants within the F<sub>2</sub> populations derived from F<sub>1</sub>1 (lanes 1-9) and F<sub>1</sub>4 (lanes 10-17).

(b) RFLP analysis of *Dra*I-digested DNA, probed with radiolabelled insert from the partial barley nitrite reductase cDNA clone pBNiR1 (Ward *et al*, 1995), from the same individual plants.

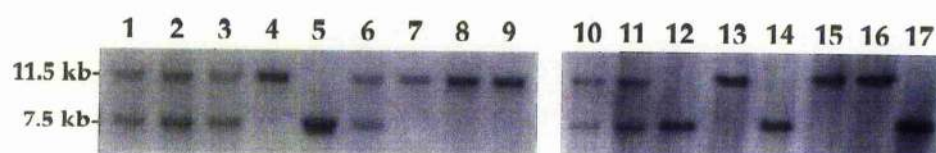
Leaf nitrite accumulation screens, DNA extraction and Southern hybridisation were performed as described in Materials and Methods. Band sizes are shown on the left.



(a)



(b)



A further fourteen seed from each of the segregating F<sub>2</sub>1 and F<sub>2</sub>4 populations were then sown, of which 10 and 9 seed germinated respectively, and were screened for leaf nitrite accumulation as described in Materials and Methods except that prior to the addition of sulfanilamide and NED to the screen, the leaf tips used in the screen were rescued and stored at -70°C for subsequent immunoblot analysis. After identification of the nitrite accumulators, the individual plants were harvested and *Dra*I-digested genomic DNA from these samples was probed with BNiR1 and is shown in Figure 5.3. Leaf tips rescued from the leaf nitrite accumulation screen were then extracted with the buffer described by Kuo *et al* (1980) and immunoblot analysis using the polyclonal anti-barley nitrite reductase antibody (Duncanson *et al*, 1992) showed that all of the leaf tips from F<sub>2</sub> Acc<sup>+</sup> plants which accumulated nitrite also lacked any detectable NiR-CRM, unlike the nitrite non-accumulating leaf tips which possessed NiR-CRM at 63kDa (Figure 5.3). All of the Acc<sup>+</sup> individuals, which lacked detectable NiR-CRM, were homozygous for the Tweed RFLP band at 11.5kb and all of the Acc<sup>-</sup> individuals, which possessed NiR-CRM, were either homozygous for the Golden Promise RFLP band at 7.5kb or were heterozygous for the RFLP bands (Figure 5.3).

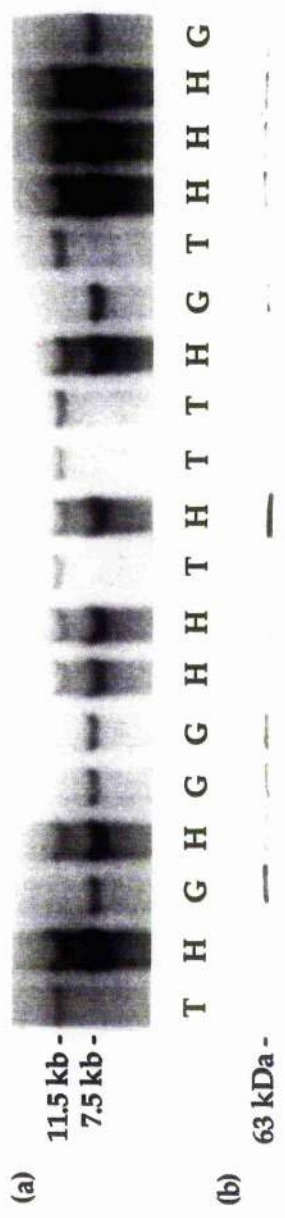
**Figure 5.3:** Cosegregation of the Tweed RFLP band with lack of nitrite reductase cross-reacting material

Cosegregation of the Tweed RFLP band at 11.5kb with lack of nitrite reductase cross-reacting material in F<sub>2</sub> individuals from the cross Golden Promise x STA3999.

(a) RFLP analysis of *Dra*I-digested DNA from individual plants within the F<sub>2</sub> populations derived from F<sub>1</sub>1 and F<sub>1</sub>4, probed with radiolabelled insert from the partial barley nitrite reductase cDNA clone pBNiR1 (Ward *et al*, 1995). T-Tweed band at 11.5kb; GP-Golden Promise RFLP band at 7.5kb; H-Heterozygous for RFLP bands. DNA extraction and Southern hybridisation were performed as described in Materials and Methods.

(b) Immunoblot analysis of nitrite reductase cross-reacting material in the leaf tissue of the same F<sub>2</sub> plants, performed using polyclonal anti-nitrite reductase antibody (Duncanson *et al*, 1992) as described in Materials and Methods.

Band sizes are shown on the left.



#### 5.2.3.2 Segregation analysis of pooled $F_2$ plants

As resources did not permit full RFLP analysis of all  $F_2$  individuals further studies were conducted using only  $Acc^+$  individuals in a batch procedure. The remaining 288  $F_{21}$  and  $F_{24}$  seed were sown and were screened for leaf nitrite accumulation after rescue of the leaf tips as described above and  $F_2$  individuals identified as nitrite accumulators were harvested individually and stored at  $-70^{\circ}C$  for subsequent DNA extraction and Southern analysis.

The leaf tips rescued from the nitrite accumulators were arranged into batches, each batch containing the leaf tips from six plants, and immunoblot analysis was performed on these batches using the polyclonal anti-barley nitrite reductase antibody (Duncanson *et al*, 1992) in order to identify any batches which contained NiR-CRM and therefore  $Acc^-$  (NiR-CRM-plus) plant(s) (Figure 5.4). In the event of a batch containing NiR-CRM, the plants within the batch could be identified and studied individually. However, in this study no batches were found to contain NiR-CRM and it was concluded that the batches contained only  $Acc^+$  plants.

Half of the remaining leaf tissue from the  $Acc^+$  individuals in these batches, which had previously been stored at  $-70^{\circ}C$ , was arranged into further batches, each batch containing the same six individuals as previously. *DraI*-digested genomic DNA from these batches was probed with BNiR1 (Figure 5.4) and showed that the batches only displayed the Tweed RFLP band at 11.5kb (Figure 5.4). A total of 72 nitrite accumulators in twelve batches of six from the 276  $F_{21}$  and  $F_{24}$  individuals which germinated were studied in this way (Figure 5.4).



**Figure 5.4: RFLP analysis and immunoblot analysis of F<sub>2</sub> pools**

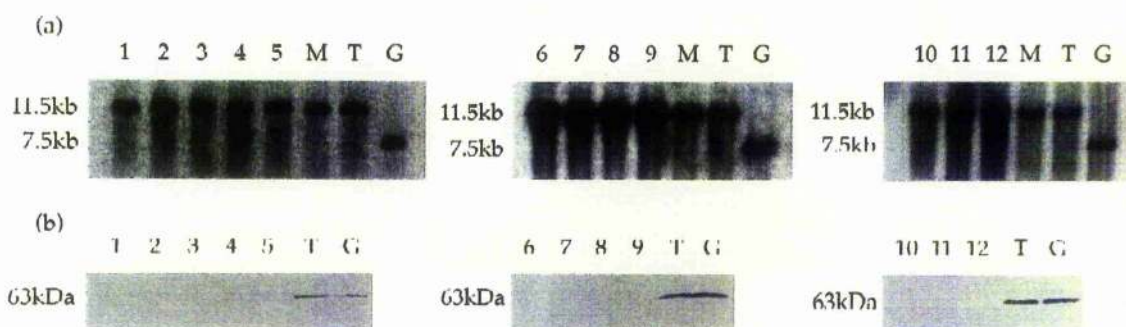
(a) RFLP analysis of *Dra*I-digested DNA from pools of six plants identified as nitrite accumulators from within the F<sub>2</sub> populations derived from F<sub>1</sub>1 (lanes 1-5) and F<sub>1</sub>4 (lanes 6-12) probed with radiolabelled insert from the partial barley nitrite reductase cDNA clone pBNiR1 (Ward *et al*, 1995).

(b) Immunoblot analysis of the same pools using polyclonal anti-nitrite reductase antibody (Duncanson *et al*, 1992).

**Abbreviations:**

T denotes DNA from wild-type cv Tweed; M denotes DNA from the cv Tweed *nir1* mutant STA3999; G denotes DNA from the wild-type cv Golden Promise.

DNA extraction, Southern hybridisation and immunoblot analysis were performed as described in Materials and Methods. Band sizes are shown on the left.





Reconstruction experiments were performed to establish the sensitivity of this batch procedure with regard to detecting single plants within a batch which are not homozygous for the Tweed RFLP band at 11.5kb. Batches were prepared from leaf tissue of 12 individuals in the ratios 10 Tweed: 2 Golden Promise, 11 Tweed:1 Golden Promise and 17 Tweed: 1 Golden Promise and *DraI*-digested DNA from these batches was probed with BNiR1 (Figure 5.5). This study demonstrates that the Golden Promise RFLP band at 7.5kb from one homozygous Golden Promise plant was detectable in a batch with 11 Tweed plants. Alternatively, the Golden Promise RFLP band from one heterozygote would be detectable in a batch with 5.5 Tweed individuals. In the event of a batch containing recombinant plants, that is,  $Acc^+$  plants possessing a Golden Promise RFLP band at 7.5kb, the unprocessed leaf tissue of individuals in the batch could have been studied further.

The data from this batch experiment, when combined with that from the analysis of F<sub>2</sub>1 and F<sub>2</sub>4 individuals (Table 5.2), show that the F<sub>2</sub> segregation ratio of 228  $Acc^-$ :84  $Acc^+$  is not significantly different at the 5% level to the Mendelian 3:1 ratio for a recessive nuclear mutation.

**Figure 5.5: Sensitivity of RFLP analysis of DNA pools**

RFLP analysis of *Dra*I-digested DNA from pools of six leaves, probed with radiolabelled insert from the partial barley nitrite reductase cDNA clone pBNiR1 (Ward *et al*, 1995).

Lanes:

Lane 1, pool of six leaves in a ratio of 5 Tweed:1 Golden Promise

Lane 2, pool of six leaves in a ratio of 11 Tweed: 1 Golden Promise

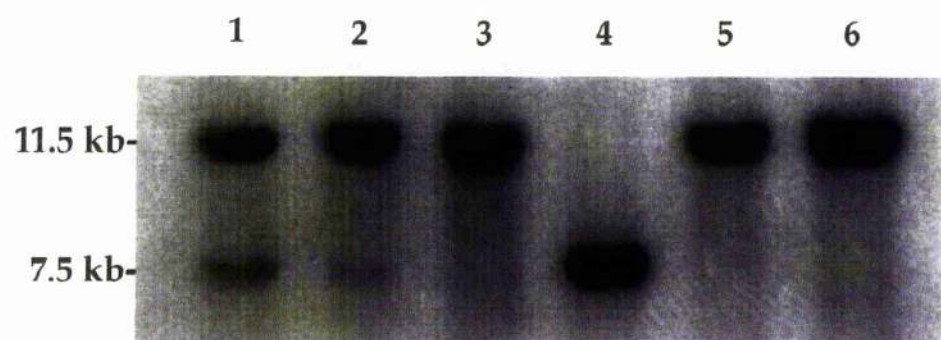
Lane 3, pool of six leaves in a ratio of 17 Tweed: 1 Golden Promise

Lane 4, wild-type cv Tweed

Lane 5, wild-type cv Golden Promise

Lane 6, Tweed *nir1* mutant STA3999

DNA extractions and Southern hybridisation were performed as described in Materials and Methods. Band sizes are shown on the left.



**Table 5.2:** Cosegregation of leaf nitrite accumulation with the Tweed RFLP band within the F<sub>2</sub> populations derived from the cross Golden Promise x STA3999

Population	No. nitrite non-accumulators	No. nitrite accumulators	$\chi^2$ (3:1)
F <sub>21</sub>	100	36	0.16
F <sub>24</sub>	128	48	0.48
TOTAL	228	84	0.61

Co-segregation of the leaf nitrite accumulation phenotype with the Tweed RFLP band at 11.5kb within the segregating F<sub>2</sub> populations derived from the F<sub>1</sub> plants F<sub>11</sub> and F<sub>14</sub>. A  $\chi^2$  value of below 3.84 indicates that the segregation ratio is not significantly different at the 5% level from the Mendelian 3:1 ratio.

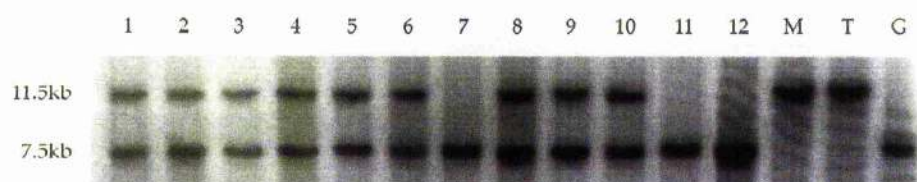
#### 5.2.4 Analysis of F<sub>3</sub> population from the cross Golden Promise x STA3999

Studies were performed to analyse the inheritance of the Acc<sup>+</sup> phenotype in the F<sub>3</sub> population derived from the cross Golden Promise x STA3999. A total of twelve Acc<sup>-</sup> individuals from the segregating F<sub>2</sub>1 and F<sub>2</sub>4 populations, hence not needed for the pooled F<sub>2</sub> mutant analysis, were replanted in compost and after one month of replanting, leaf tissue samples were collected from the twelve plants. *Dra*I-digested DNA from these samples was probed with BNiR1 (Figure 5.6). The twelve individuals were then allowed to self-pollinate and produce F<sub>3</sub> progeny of which a small samples were screened for leaf nitrite accumulation (Table 5.3). These results show that all heterozygous plants produced a progeny segregating for leaf nitrite accumulation in an approximate 3:1 ratio of Acc<sup>-</sup>:Acc<sup>+</sup>, whereas all plants homozygous for the Golden Promise RFLP band gave rise to non-segregating progeny. Thus, leaf nitrite accumulation only occurs in populations containing the STA3999 RFLP band at 11.5kb.

**Figure 5.6: RFLP analysis of F<sub>2</sub> nitrite non-accumulating individuals**

RFLP analysis of *Dra*I-digested DNA from Acc<sup>-</sup> individuals within the F<sub>2</sub> populations derived from F<sub>1</sub>1 (lanes 1-6) and F<sub>1</sub>4 (lanes 7-12), probed with the partial barley nitrite reductase cDNA clone pBNiR1 (Ward *et al*, 1995).

DNA extractions and Southern hybridisation were performed as described in Materials and Methods. Band sizes are shown on the left.





**Table 5.3:** Inheritance of leaf nitrite accumulation within the F<sub>3</sub> progeny of F<sub>2</sub> nitrite non-accumulating individuals

Population	No. nitrite non-accumulators	No. nitrite accumulators	$\chi^2$ (3:1)
1	9	3	0
2	10	4	0.09
3	11	3	0.09
4	10	4	0.09
5	9	4	0.23
6	9	3	0
7	13	0	-
8	12	2	0.85
9	10	4	0.09
10	8	3	0.03
11	13	0	-
12	11	0	-

Inheritance of leaf nitrite accumulation within the F<sub>3</sub> populations derived from Acc<sup>-</sup> individuals within the segregating F<sub>2</sub> populations derived from the cross Golden Promise x STA3999. A  $\chi^2$  value of below 3.84 indicates that the segregation ratio is not significantly different at the 5% level from the Mendelian 3:1 ratio. Leaf nitrite accumulation screens were performed as described in Materials and Methods.

## 5.3 DISCUSSION

### 5.3.1 Identification of an RFLP between barley cv Tweed and barley cv Golden Promise

Previous studies on barley (J.L. Wray, University of St Andrews, UK; personal communication) demonstrated an RFLP between the barley wild-type cv Tweed (major hybridising band at 11.5kb) and the barley wild-type cv Golden Promise (major hybridising band at 7.5kb) when *DraI*-digested genomic DNA was probed with radiolabelled insert from the partial barley nitrite reductase cDNA clone pBNiR1 (Ward *et al*, 1995). It is demonstrated here that there is no RFLP detected between wild-type cv Tweed and the Tweed *nir1* mutant STA3999 (Figure 6.1). Crossing of the wild-type cv Golden Promise to the Tweed *nir1* mutant STA3999 (J.L. Wray, University of St Andrews, UK; personal communication) has led to the isolation of F<sub>2</sub> populations of barley cosegregating for the Tweed RFLP band at 11.5kb and the *nir1* phenotype (leaf nitrite accumulation after treatment with nitrate/lack of detectable nitrite reductase cross-reacting material), hence allowing an estimation of the recombination frequency between the *Nir1* locus and the nitrite reductase apoprotein gene, *Nii*. This frequency can be used to calculate the map distance between the two loci.

### 5.3.2 Analysis of the F<sub>1</sub> population from the cross Golden Promise x STA3999

Four putative F<sub>1</sub> plants were available from the cross Golden Promise x STA3999. Two of these putative F<sub>1</sub> plants were shown to be heterozygous for the RFLP (Figure 6.1) and produced progeny which segregated for the *nir1* phenotype in a 3:1 ratio of Acc<sup>-</sup>:Acc<sup>+</sup> plants (Table 5.1). The remaining

two putative F<sub>1</sub> plants were homozygous for the Golden Promise RFLP band (Figure 5.1) and produced progeny which did not segregate for the *nir1* phenotype (Table 5.1). It was concluded that the putative F<sub>1</sub> plants homozygous for the Golden Promise RFLP band were products of self-pollination of the female (pollen recipient) Golden Promise parent and these plants were not studied further.

### 5.3.3 Analysis of the F<sub>2</sub> population from the cross Golden Promise x STA3999

Plants within the segregating F<sub>2</sub> populations derived from the cross Golden Promise x STA3999 were examined both individually (Figures 5.2-5.3) and in batches (Figure 5.4) for cosegregation of the Tweed RFLP band at 11.5kb and the *nir1* phenotype. Data from the 36 F<sub>2</sub> plants analysed individually combined with the data from the 72 pooled F<sub>2</sub> mutants has unambiguously identified 84 F<sub>2</sub> plants with the mutant parental phenotype from a total population of 312 F<sub>2</sub> plants. As no recombination was found between the homozygous Golden Promise/heterozygous RFLP genotype and the *nir1* phenotype, one of the possible recombinant classes was 0. The remaining plants were not separated into the other recombinant and wild-type parental classes but as no recombinant was found in the smaller sample of 36 F<sub>2</sub> plants analysed in detail, it was assumed that none existed in the larger sample. This suggests that the two loci, *Nir1* and *Nii*, are either allelic or very tightly linked. If the latter applies then some idea of the proximity of the two loci can be estimated by postulating that one of the 312 F<sub>2</sub> plants was recombinant, in which case the loci would be  $0.3 \pm 0.3\text{cM}$  apart (W.T.B Thomas, SCRI, Invergowrie, UK; personal communication).

#### 5.3.4 Analysis of the F<sub>3</sub> population from the cross Golden Promise x STA3999

Study of inheritance of the leaf nitrite accumulation phenotype within F<sub>3</sub> populations derived from the cross Golden Promise x STA3999 (Figure 6.6; Table 6.3) shows that only F<sub>2</sub> plants heterozygous for the RFLP produce F<sub>3</sub> progeny segregating for leaf nitrite accumulation and segregation ratios were not significantly different at the 5% level to the Mendelian ratio of 3:1 for a recessive nuclear mutation. This data supports the contention that the *Nir1* locus and the *Nii* gene are tightly-linked and probably allelic.

#### 5.3.5 Identity of the *Nir1* locus

Since the *nir1* mutant STA3999 has the phenotypic characteristics expected of a mutant defective in the nitrite reductase apoprotein gene (Duncanson *et al*, 1993; Chapter 4) and no recombinants were found in this study, it is likely that the *Nir1* gene locus is allelic to the *Nii* gene locus. If this is the case then there are several possibilities as to the location of the *nir1* mutation within the *Nii* gene. The *nir1* mutants produce *nii* transcript of wild-type size (2.3kb) and at approximately wild-type levels, hence the *nir1* mutation most likely affects translation of the nitrite reductase transcript. Several mutations within the *Nii* gene could be expected to produce such an effect: mutations which have produced a stop codon in the 5' coding region of the *nii* mRNA, so the protein product is not detectable, mutations which have altered the start codon thus preventing translation; mutations affecting the ribosomal binding of the mRNA; mutations leading to the synthesis of a protein product which is particularly susceptible to the cell's degradation machinery. In order to distinguish between these possibilities it will be necessary to establish the full-length nitrite reductase cDNA (hence mRNA)

sequences of both the *nir1* mutant and its wild-type cultivar for comparative purposes.

## CHAPTER 6

### Isolation of a Barley Nitrite Reductase cDNA Clone

## 6.1 INTRODUCTION

The complete or partial nucleotide sequence of a number of higher plant nitrite reductase cDNAs and genes has been determined (Table 1.1 in Chapter 1) and comparison of the amino acid sequences deduced from these (Figure 6.1) shows that the nitrite reductase protein is well conserved among higher plants. Pairwise nitrite reductase amino acid sequence similarities are shown in Table 6.1 and range between 83-99% for the apoproteins shown, the least similar being birch and barley (83% over 71 amino acids) and the most similar being the tobacco *nir-1* and *nir-3* proteins (99% over 456 amino acids).

Structure-function relationships within the higher plant nitrite reductase protein have been deduced by amino acid sequence comparison with *Escherichia coli* sulphite reductase (EC 1.8.1.2) (*cysI*; Ostrowski *et al*, 1989), another sirohaem/Fe<sub>4</sub>S<sub>4</sub> centre-containing enzyme (Figure 6.1). High sequence similarity was found between the nitrite reductase protein and the *cysI* protein at positions 443-501 of the alignment in Figure 6.1. This region contains four cysteine residues at positions 445, 451, 485 and 489 of the alignment (corresponding to positions 473, 479, 514 and 518 in the spinach sequence) which bind the sirohaem/Fe<sub>4</sub>S<sub>4</sub> centre at the reducing site of the enzyme (Siegel and Wilkerson, 1989). The bridging ligand between the sirohaem and the Fe<sub>4</sub>S<sub>4</sub> centre has been assigned to either cys445 or cys451 of the alignment (cys473 or cys479 of the spinach sequence). Friemann *et al* (1992a) compared the deduced amino acid sequences of maize, spinach and birch nitrite reductase to the amino acid sequence of spinach ferredoxin-NADP reductase (EC 1.18.1.2) and identified the presence of a short conserved sequence at the N-terminus (positions 95-120 in the alignment in Figure 6.1) containing a cluster of 5-6 positively charged amino acids. It was postulated that these amino acids participate in binding ferredoxin.



Higher plant nitrite reductases are synthesised as precursor proteins with an N-terminal extension, the transit peptide, which acts to target the nitrite reductase protein to and within the chloroplast or plastid and is then cleaved from the mature protein on entry. Amino acid sequence comparisons between transit peptides of higher plant nitrite reductases and transit peptides of other nuclear-encoded chloroplast-acting enzymes such as the light-harvesting chlorophyll a/b-protein II and the small subunit of ribulose 1,5-bisphosphate carboxylase show little similarity (Back *et al*, 1988), although other studies suggest that different precursor proteins share components in the chloroplast envelope transport apparatus even when they are sorted to different chloroplastic compartments (Perry *et al*, 1991). Transit peptide functional domains have been difficult to identify. Studies of the ribulose 1,5-bisphosphate carboxylase small subunit (Perry *et al*, 1991) suggest that the central region of the transit peptide mediate binding to the chloroplast surface, whereas the N-terminal and C-terminal regions of the transit peptide are more important for translocation across the envelope. Archer and Keegstra (1993) showed that removal of a conserved arginine residue in the C-terminal region of the ribulose 1,5-bisphosphate carboxylase small subunit impaired processing but not necessarily import rates, while substituting amino acids of low amphiphilic potential for other original residues in this region affected import rate but not processing, suggesting that the C-terminal region of the transit peptide is important for translocation of the protein across the chloroplast envelope and proper processing of the precursor protein. Studies of the transit peptide of ferredoxin (Vanthof and Dekruiff, 1995) suggest that ATP is involved in translocation and that initial binding is facilitated by transit peptide-lipid interactions.

The alignment in Figure 6.1 begins at the position of the putative first amino acid residue of the mature spinach nitrite reductase protein (Back *et al*, 1988).

The nucleotide sequences of the nitrite reductase genes from spinach, tobacco, *Arabidopsis* and bean have been characterised (Table 1.1) and all of these contain three introns in the same positions (corresponding to positions 104, 222 and 319 of the alignment in Figure 6.1). Intron lengths, however, differ between species, being 531, 93 and 93 nucleotides in the spinach nitrite reductase gene; 500, 550 and 150 nucleotides in the tobacco nitrite reductase gene; 196, 81 and 77 nucleotides in the *Arabidopsis* nitrite reductase gene and 651, 221 and 509 nucleotides in the bean nitrite reductase gene respectively. As described in Chapter 1, the characterisation of higher plant nitrite reductase genomic DNA has proved of great importance as it has made possible the analysis of nitrite reductase promoter regions through the study of transgenic plants.

The number of nitrite reductase genes differs between higher plant species. Copy number determinations suggest that some species such as spinach (Back *et al*, 1991), birch (Friemann *et al*, 1992a) and barley (A.Sherman and J.L.Wray, unpublished) possess a single nitrite reductase gene per haploid genome whilst other species possess multiple copies. Maize possesses at least two gene copies but only a single cDNA species has been characterised (Lahners *et al*, 1988). In tobacco, a combination of cDNA cloning and Southern hybridisation demonstrates the presence of four genes (Kronenberger *et al*, 1993). Two of these genes (*nir-1* and *nir-2*) are believed to be derived from *Nicotiana tomentosiformis* and two (*nir-3* and *nir-4*) from *Nicotiana sylvestris*, the two ancestral progenitors of *Nicotiana tabacum*.

It has been established by RFLP mapping (Chapter 5) that the *Nir1* locus in barley is within 0.3 cM of, and probably in, the nitrite reductase apoprotein gene, *Nii*. It is possible that the *nir1* phenotype (leaf nitrite accumulation/NiR-CRM-minus) is due to a mutation in a tightly-linked but distinct regulatory locus. This is unlikely as the four *nir1* mutants synthesise leaf *nii* transcript of wild-type size and at approximately wild-type levels in

response to treatment with nitrate in the light (Chapter 4), suggesting that the *nir1* mutation is not present in a regulatory locus as defects in the signal transduction pathway through which light, nitrate and the plastidic factor act to up-regulate nitrite reductase transcript would lead to a lack of detectable *nii* transcript. The *nir1* phenotype is most likely the effect of a mutation in the *Nii* gene which either affects translation of the *nii* transcript or stability of the nitrite reductase protein. That is, the *nir1* mutations are not in the promoter or intron regions.

In order to confirm the identity of the *Nir1* locus as the nitrite reductase apoprotein gene and to establish the intragenic location of the *nir1* mutations, the nucleotide sequences of the nitrite reductase cDNAs from the *nir1* mutants and from the wild-type cultivars from which they were isolated must be determined for comparative purposes. Due to the availability of plant material, the *nir1* mutant STA3999 and its wild-type cv Tweed were selected for this study and this chapter describes attempts to isolate a full-length cDNA clone coding for nitrite reductase from barley cv Tweed, the wild-type cultivar from which the *nir1* mutant STA3999 was isolated.

**Table 6.1:** Deduced amino acid sequence similarities between higher plant nitrite reductases

Pairwise similarities between amino acid sequences deduced from higher plant nitrite reductase cDNAs and genes were determined using the GAP program from the University of Wisconsin GCG v8.1-UNIX sequence analysis software package at Daresbury, UK. References for the cDNA sequences used are given in Table 1.1 (Chapter 1).



## Species

	Bean	Arabidopsis	Spinach	Tobacco nir-2	Birch	Tobacco nir-3	Tobacco nir-1	Rice	Maize
Barley	85%	84%	89%	Does not overlap	83%	90%	90%	92%	94%
Maize	87%	87%	86%	87%	87%	88%	87%	92%	
Rice	85%	86%	83%	89%	86%	89%	87%		
Tobacco nir-1	90%	88%	87%	94%	89%	99%			
Tobacco nir-3	92%	92%	94%	94%	92%				
Birch	91%	88%	90%	93%					
Tobacco nir-2	91%	91%	94%						
Spinach	88%	88%							
Arabidopsis	89%								

**Figure 6.1:** Amino acid sequence alignment between higher plant nitrite reductases (2 pages)

Amino acid sequences were deduced from nitrite reductase cDNA and gene sequences (References given in Table 1.1). Sequences were aligned using the PILEUP program from the University of Wisconsin GCG v8.1-UNIX sequence analysis software package. Gaps (dots) were introduced where necessary to maximise homology. Residues present in 50% or more of the sequences at any given position are shown in bold type. Residues common to all sequences at any given position are shown in the CONSENSUS sequence. 1 indicates the start of the mature spinach protein (Back *et al*, 1988). Positions of the introns in spinach, tobacco, *Arabidopsis* and bean are marked with an asterisk (\*). The alignment further includes amino acid sequence derived from *Escherichia coli* sulphite reductase (*cysI*) (Ostrowski *et al*, 1989). Residues common to both sulphite reductase and nitrite reductase sequences are underlined. In the sulphite reductase sequence the position of four additional residues not found in nitrite reductase is marked by an oblique (/). The four conserved cysteine residues common to nitrite reductase and sulphite reductase which are implicated in sirohaem/Fe<sub>4</sub>S<sub>4</sub> binding are denoted by ‡.

1 50

Maize 1 PORTGRARAAVSVPPPAGEQVPTERIEPRVEERAGGYWLVKKEKYRAGLNPQEKVKLEKEPMALFMEGGIO

Rice MASSASLQRLFPYPHAAASRCRPPGVRARPVQSSTVSAPSSSTPAADAEVSAERLEPRVEQREGRYWLVKKEKYRTGLNPQEKVKLEKEPMALFMEGGIK

Tobacco nir1 SIKFLAPSLPNPARFKNVAVKLHATPPSVAAAPAGAPEVAERLEPRVEEKDG.YWILKEQFRKGINPQEKVKLEKQPMKLFMEGGIE

Birch MSSLSVRFLSPPLFSSTPAWPRTGLAATQAVPPVVAEVDAGRLERPRVEEREG.YWLVKKEKFRKGINPQEKVKLEKQPMKLFMEGGIE

Spinach MASLPVNKIIPSSTTLSSNNRRNNSSIRCQKAVSPAETAASVSDAARLEPRVEERDG.FWLVKKEEFRSGINPAEKVKIEKQPMKLFIEDGIS

Arabidopsis MTSFSLTFTSPLLPSSSTPKRSLVLAQAQTAPAEASTASVDADRTLEPRVELKDG.FFVLKKEKFRKGINPQEKVKIEKQPMKLFMEGGIE

Bean MSSFSVRFLAPPCCPTSRSKTLLCASPTAA.PAASEAVEASRLERPRVEERDG.FWLVKKEEYRGGISPQEKVKLEKQPMKLFMEGGIE

CONSENSUS S LEPRVE G LKE R G P EK K PM LF E GI

100 \* 150

Maize DLARVPMQIDAAKLTK.DDVVRLKWLGLFHRKHKHQYGRFMMRLKLPNGVTTSEQTRYLASVIEAYGADGCAADVTTTRQNWQIRGVTLPDVPAILDGLRA

Rice ELAKMPEIEADKLSK.EDIDVRLKWLGLFHRKHKHQYGRFMMRLKLPNGVTTSEQTRYLASVIEAYGEGCADVTTTRQNWQIRGVTLPDVPAILDGLNA

Tobacco nir1 ELAKIPIEIDQSKLTKEDDIDVRLKWLGLFHRKKNQYGRFMMRLKLPNGVTTSAQTRYLASVIRKYKEGCADIITTRQNWQIRGVTLPDVPAILKGLAE

Tobacco nir3 MMRLKLPNGVTTSAQTRYLASVIRKYKEGCADIITTRQNWQIRGVTLPDVPAILKGLAE

Tobacco nir2 GDVTTTRQNWQIRGVTLPDVPAILKGLDE

Birch DLAKMSLEIDKDKISK.SDIDVRLKWLGLFHRKHKHQYGRFMMRLKLPNGVTTSAQTRYLASVIRKYKGDGCAADVTTTRQNWQIRGVTLPDVPAILKGLDE

Spinach DLATLSMEEVDKSKHNK.DDIDVRLKWLGLFHRKHKHQYGRFMMRLKLPNGVTTSEQTRYLASVIRKYKGDGCAADVTTTRQNWQIRGVTLPDVPAILKGLDE

Arabidopsis ELAKMSLEELDSEKSSK.DDIDVRLKWLGLFHRKHKHQYGRFMMRLKLPNGVTTSAQTRYLASVIRKYGEDGCAADVTTTRQNWQIRGVTLPDVPAILKGLAS

Bean DLAKMSLEIEESSKHTK.DDIDVRLKWLGLFHRKHKHQYGRFMMRLKLPNGVTTSSQTRYLASVIRKYKGDGCAADVTTTRQNWQIRGVTLPDVPAILKGLAE

CONSENSUS LA E K K D DVRLKWLGLFHRK YG FMMRLKLPNGVTT S QTRYLASVI YG GC D TTRQNWQIRGV L DVP I GL

200 \* 250

Maize VGLTSLQSGMDNVRNPVGNPLAGVDPHEIVDTRPYTNLLSSYVTNNSQGNPTITNLPKRKNVVCVIGSHDLYEHPHINDLAYMPA.VKDGEFGFNLLVGGF

Rice VGLTSLQSGMDNVRNPVGNPLAGIDPDEIVDTRSYTNLLSSYITSNFQGNPTITNLPKRKNVVCVIGSHDLYEHPHINDLAYMPA.VKGKFGFNLLVGGF

Tobacco nir1 VGLTSLQSGMDNVRNPVGNPLAGIDPEEIVDTGTPYTNLLSQFITGNSRGNPAVSNLPKRKNPCVVGSHDLYEHPHINDLAYMPA.TKDGRFGFNLLVGGF

Tobacco nir3 VGLTSLQSGMDNVRNPVGNPLAGIDPEEIVDTRPYTNLLSQFITGNSRGNPAVSNLPKRKNPCVVGSHDLYEHPHINDLAYMPA.TKDGRFGFNLLVGGF

Tobacco nir2 VGLTSLQSGMDNVRNPVGNPLAGIDPHEIVDTRPYTNLLSQYVTANFRGNPAVITNLPKRKNVVCVIGSHDLYEHPHINDLAYMPA.SKDGRFGFNLLVGGF

Birch VGLTSLQSGMDNVRNPVGNPLAGIDITHEIVATRPYNLLSQFITANSRGNLAFTNLPKRKNVVCVVGSHDLFEHPHINDLAYMPA.IKDGRFGFNLLVGGF

Spinach VGLTSLQSGMDNVRNPVGNPLAGIDPHEIVDTRPYTNLLSQFITANSRGNLSITNLPKRKNPCVIGSHDLYEHPHINDLAYMPA.TKNKFGFNLLVGGF

Arabidopsis VGLTSLQSGMDNVRNPVGNPLAGIDPEEIVDTRPYTNLLSQFITANSQGNPDFTNLPKRKNVVCVVGTHDLYEHPHINDLAYMPA.NKDGRFGFNLLVGGF

Bean VGLTSLQSGMDNVRNPVGNPLAGIDPDEIVDTRPYTNLLSQFITANSGLGNPAMSNLPKRKNVVCVVGSHDLFEHPHINDLAYMPANNKDGFRFGFNLLVGGF

CONSENSUS VGLTSLQSGMDNVRN VGNP AG D EIV T NL S T N GN NLPRKN CV G HDL EHPHINDLAYMPA K G FGFNLLVGGF



Maize ISPKRWAEALPLDAWAGDDVVPVCKAILEAYRDLGSRGNRQKTRMMWLIDELGMEVFRSEVEKRMPPNGVLERAAPEDLVDKRWRDYLGVHPQKQEG  
 Rice ISPKRWEEALPLDAWPGDDIIPVCKAILEAYRDLGTRGNRQKTRMMWLIDELGMEAFRSEVEKRMPPNGVLERAAPEDLVKKWRDYLGVHPQKQEGM  
 Tobacco nir1 FSAKRCDDEAIPLDWVPADDVVPVCKAILEAFRDLGFRGNRQKCRMMWLIDELGVGEFRAEVEKRMPPQQLERASPEDLVQKQWRDYLGVHPQKQEGY  
 Tobacco nir3 FSAKRCDDEAIPLDWVPADDVVPVCKAILEAFRDLGFRGNRQKCRMMWLIDELGVGEFRAEVEKRMPPQQLERASPEDLVQKQWRDYLGVHPQKQEGY  
 Tobacco nir2 FSPKRCAEAVPLDAWVPADDVVPVCKAILEAYRDLGTRGNRQKTRMMWLIDELGVGEFRAEVEKRMPPQQLERASPEDLVQKQWRDYLGVHPQKQEGY  
 Birch FSPKRCAEAVPLDAWVPADDVVPVCKAILEAYRDLGTRGNRQKTRMMWLIDELGVGEFRAEVEKRMPPQQLERASPEDLVQKQWRDYLGVHPQKQEGY  
 Spinach FSIKRCDEAIPLDWVSAEDVVPVCKAMLEAFRDLGFRGNRQKCRMMWLIDELGMEAFRGEVEKRMPEQVLERASSEELVQKQWRDYLGVHPQKQEGY  
 Arabidopsis FSPKRCDEAIPLDWVPADDVPLCKAILEAYRDLGTRGNRQKTRMMWLIDELGVGEFRAEVEKRMPPNGKLERGSSDELVNKQWRDYLGVHPQKQEGY  
 Bean FSAKRCAEALPLDAWVSAEDVVPVCKAILEAYRDLGFRGNRQKTRMMWLIDELGMEVFRSEVEKRMAGQLERA.QEDLVKKQWRDYLGVHPQKQEGY  
 CONSENSUS S R EA PLDAWV D CKA LEA RDLG RGNRQK RMMWL DELG E FR EV KRM L R E L K W RR GV PQKQ G

Maize SYVGLHVPVGRVQAADMFEALRLADEYGTGELRLTVEQNIIVLPVNSNERLDALLAEPLLEQORLSRPSMLLRGLVACTGNQFCGQAIETKARALQVAR  
 Rice SYVGLHVPVGRVQAADMFEALRLADEYGSGEIRLTVEQNIIVIPNVKNEKVEALLSEPLL.QKFSQPSPSLLKGLVACTGNQFCGQAIETKQALLVTS  
 Tobacco nir1 SFIGLHIPVGRVQADDMDELARLADYGSGEIRLTVEQNIIPNIENSKIEALLKEPVL.ST.FSPDPPILMKGLVACTGNQFCGQAIETKARSLMITE  
 Tobacco nir3 SFIGLHIPVGRVQADDMDELARLADYGSGEIRLTVEQNIIPNIENSKIEALLKEPVL.ST.FSPDPPILMKGLVACTGNQFCGQAIETKARSLKITE  
 Tobacco nir2 SFVGLHIPVGRVQADDMDELARLADYGSGEIRLTVEQNIIPNVENSKIESLINEPLL.KNRFSTNPPILMKNLVACTGNQFCGQAIETKARSMKITE  
 Birch SYVGLHIPVGRVQADDMDELARLADYGSGEIRLTVEQNIIPNIENSKIEALLKEPVL.KDRFSPEPPILMKGLVACTGNQFCGQAIETKARALKVTE  
 Spinach SFVGLHIPVGRVQADMEELARLADYGSGEIRLTVEQNIIPNVENSKIESLINEPLL.KERYSPPEPPILMKGLVACTGSQFCGQAIETKARALKVTE  
 Arabidopsis SFVGLHIPVGRVQADDMDELARLADYGSGEIRLTVEQNIIPNVETSKTEALLQEPFL.KNRFSPPEPPILMKGLVACTGSQFCGQAIETKARALKVTE  
 Bean SYVGLHIPVGRVQADMEDELALALADEYGSGEIRLTVEQNIIPNVDSNKLKLEALLQEPFL.KHKFSPEPPILMKGLVACTGNQFCGQAIETKARALKVTE  
 CONSENSUS S G H PVGR QA M ELA AD YG GE RLTVENI PN LL EP L S P L LVACTG QFCGQAIETK R  
 E.coli cysI MACVSFPTCPLAMAEAEERFLPSFTD  
 + +

Barley DIGFMGCLTKNSSKIVEAADIFVGGRIASDSLHTGVYKAVPCEDLVPIVADLLVERFGAVPREREDEE  
 Maize DIGFMGCLTKDSDGKIVEAADIFVGGRVGSDSHLADYRKSVPCKDLVPIVADLLVERFGAVPREREDEE  
 Rice QVEKLVSPRAVRMHWTCGNPCNSCGQVQVADIGFMGCLTKDSAGKIVEAADIFVGGRVGSDSHLAGAYKSVPCDELAPIVADILVERFGAVPREREDEE  
 Tobacco nir1 EVQRQVSLTRPVRMHWTCGNPCNTCAQVQVADIGFMGCLTRDKNGKTVEGADVFLGGRIGSDSHLGEVYKAVPCDDLVPLVVDLLVNNFGAVPREREDEE  
 Tobacco nir3 EVQRQVSLTKPVRMHWTCGNPCNTCAQVQVADIGFMGCLTRDKNGKTVEGADVFLGGRIGSDSHLGEVYKAVPCDDLVPLVVDLLVNNFGAVPREREDEE  
 Tobacco nir2 EVQRQVSLTKPVRMHWTCGNPCNSCGQVQVADIGFMGCMARDENKPKCEGAAVFLGGRIGSDSHLGNLYKKGVPCKNLVLVVDLLVKKHFGAVPRERESEED  
 Birch EVQRQVAVTRPVRMHWTCGNPCNSCGQVQVADIGFMGCMARDENKPKCEGAAVFLGGRIGSDSHLGNLYKKGVPCKNLVLVVDLLVKKHFGAVPRERESEED  
 Spinach EVQRQVSVTRPVRMHWTCGNPCNSCGQVQVADIGFMGCMARDENKPKCEGAADVFGVGGIGSDSHLGDYIKKAVPCDLVVPVAEILINQFGAVPREREAE  
 Arabidopsis EVERLVSVPRI RMHWTCGNPCNTCGQVQVADIGFMGCLTRGEEKPVGEGADVFGGRIGSDSHLGEIYKGVRTVELVPLVAEILIKEFGAVPREREENED  
 Bean EVERQVAVTRPVRMHWTCGNPCNTCGQVQVADIGFMGCMARDENKATEGVDFLGGIGSDSHLAEIYKGVPCKNLVPVVDLLVKKHFGAVQNRREVED  
 CONSENSUS V V RMHWTCGN C QVQVADIGFMG GK E GGR SDSH Y K V L P V L FGAV R REE E  
 E.coli cysI KYEAILEK/EHIVMRVTGCPNGCGRAMLAELGLV  
 + +

## 6.2 RESULTS

### 6.2.1 Preparation of a cDNA library from barley cv Tweed

#### 6.2.1.1 *Isolation of poly A<sup>+</sup> RNA*

In order that the cDNA library could also be used for studies involving the sulphate assimilation pathway, poly A<sup>+</sup> RNA was isolated from different barley tissues under various growth conditions. Total RNA was extracted from 5g of leaf tissue and 8g of root tissue from 7 day-old, green barley cv Tweed plants treated with 25mM potassium nitrate in the light for 8 hours and from 5g of leaf tissue and 8g of root tissue from 7 day-old sulphur-deprived green barley cv Tweed plants treated with 25mM potassium nitrate for 18 hours in the light. Total RNA yields, as determined by spectrophotometric analysis, are shown in Table 6.2a. Minigel analysis of 1µg of each of these preparations is shown in Figure 6.2a. Although there were small amounts of genomic DNA present in the total RNA samples, it was anticipated that these would be removed by the poly A<sup>+</sup> isolation step.

Poly A<sup>+</sup> RNA was isolated from each of the four total RNA samples using QIAGEN<sup>TM</sup> oligotex-dT columns and yields within the expected range (1-5% of total RNA) were obtained (Table 6.2b). Purity of the poly A<sup>+</sup> preparations was assessed by determination of the OD<sub>260</sub>/OD<sub>280</sub> ratio (Table 6.2b), and the poly A<sup>+</sup> preparations displayed an OD<sub>260</sub>/OD<sub>280</sub> ratio in the range 1.93-1.97. Minigel analysis of 0.5µg of each of the poly A<sup>+</sup> RNA samples (Figure 6.2b) demonstrated a poly A<sup>+</sup> RNA size range up to approximately 6kb. For the cDNA library poly A<sup>+</sup> RNA template, an approximate 4µg amount of each poly A<sup>+</sup> sample was combined and this pool gave an OD<sub>260</sub>/OD<sub>280</sub> ratio of 1.95, and a final yield of 13.33µg. Minigel analysis of 0.33µg of the poly A<sup>+</sup> template is shown in Figure 6.3.

**Table 6.2: Spectrophotometric analysis of RNA from barley cv Tweed**

(a) Yields of total RNA extracted from 5g of leaf tissue and 8g of root tissue of 7-day-old, green barley cv Tweed plants and (b) the yields and purity of poly A<sup>+</sup> RNA isolated from the total RNA samples described in (a). Extraction of total RNA, isolation of poly A<sup>+</sup> RNA and spectrophotometric analysis were performed as described in Materials and Methods.

Abbreviations: -S+N, plants grown in the absence of sulphate and treated with 25mM nitrate in the light for 18 hours; +S+N, plants grown in the presence of sulphate and treated with 25mM nitrate in the light for 8 hours.

(a)

Tissue	Growth Conditions	Total RNA yield
Leaf	-S+N	1.02 mg
	+S+N	1.35 mg
Root	-S+N	0.80 mg
	+S+N	1.10 mg

(b)

Tissue	Growth Conditions	OD <sub>260</sub>	OD <sub>280</sub>	OD <sub>260</sub> / OD <sub>280</sub> Ratio	Poly A <sup>+</sup> RNA yield
Leaf	-S+N	0.280	0.141	1.98	11.7 µg
	+S+N	0.312	0.158	1.97	13.0 µg
Root	-S+N	0.240	0.123	1.97	10.0 µg
	+S+N	0.254	0.131	1.93	10.6 µg

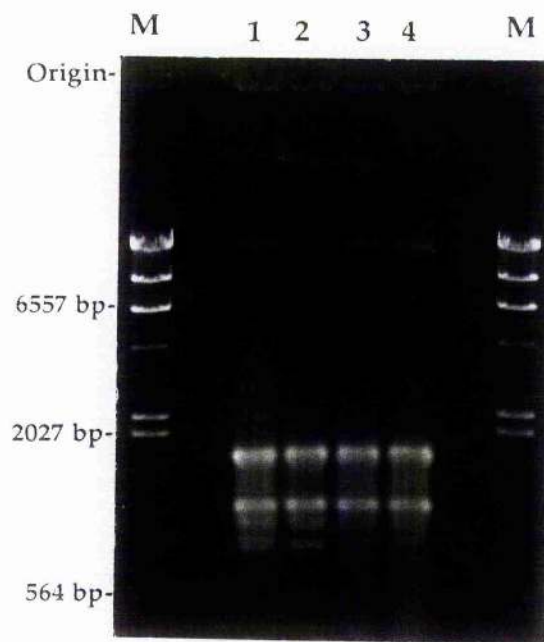
**Figure 6.2:** Minigel analysis of RNA from the leaf and root of barley cv Tweed

Minigel analysis of (a) 1 $\mu$ g of total RNA from the leaf (lane 1) and root (lane 3) of 7 day-old, green plants grown in the absence of sulphate and treated with 25mM nitrate in the light for 18 hours and from the leaf (lane 2) and root (lane 4) of 7 day-old, green plants grown in the presence of sulphate and treated with 25 mM nitrate for in the light for 8 hours and (b) 0.5 $\mu$ g of poly A<sup>+</sup> RNA isolated from the total RNA samples described in (a).

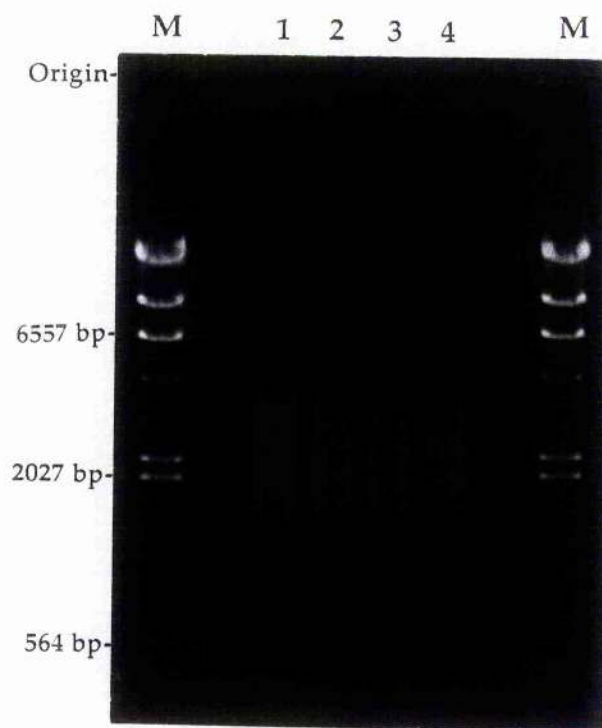
Total RNA extraction, poly A<sup>+</sup> RNA isolation and minigel analysis were performed as described in Materials and Methods.

$\lambda$ HindIII DNA markers are shown (lane M) and marker sizes are displayed on the left.

(a)



(b)

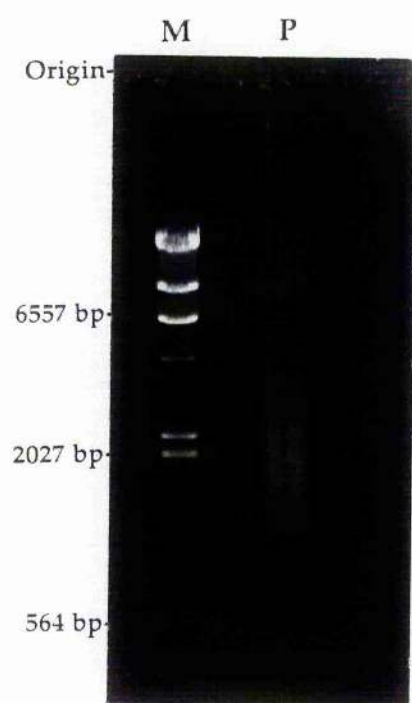


**Figure 6.3: Minigel analysis of pooled poly A<sup>+</sup> RNA**

Minigel analysis of 0.33 $\mu$ g of a pooled poly A<sup>+</sup> RNA sample (lane P) comprising a 1:1:1:1 mixture of the four poly A<sup>+</sup> RNA samples described in Figure 6.2b. Minigel analysis was performed as described in Materials and Methods.

$\lambda$ HindIII markers (lane M) and marker sizes are shown on the left).





#### 6.2.1.2 Construction of the cDNA library

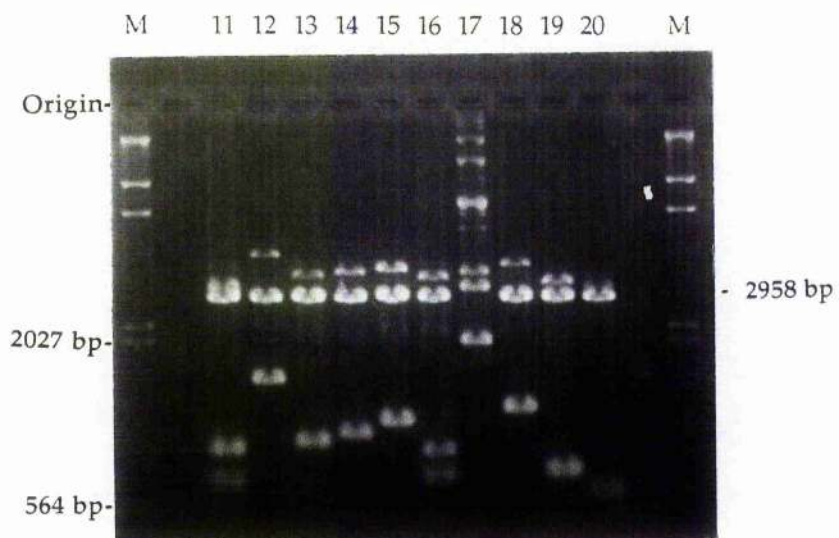
Construction of a Uni-ZAP<sup>TM</sup> XR cDNA library in  $\lambda$ ZAPII, using the remaining 13 $\mu$ g of pooled barley cv Tweed poly A<sup>+</sup> RNA as the template for first-strand cDNA synthesis, was performed by the custom cDNA library service at STRATAGENE, UK to the specifications described in Materials and Methods. The amplified cDNA library was provided at a titre of  $1 \times 10^{10}$  pfu/ml which was verified as described in Materials and Methods.

Quality of the cDNA library was assessed as the average cDNA insert size. The average insert size was estimated by mass excision of the cDNA library and subsequent minigel analysis of *Eco*RI/*Xho*I-digested plasmids from twenty randomly-selected colonies (Figure 6.4) and was found to be 1256bp (Table 6.3).

**Figure 6.4: Minigel analysis of cDNA inserts from the barley cv Tweed cDNA library**

Minigel analysis of cDNA inserts isolated by EcoRI/XhoI digestion of the plasmids from twenty randomly-selected bacterial colonies (lanes 1-20) resulting from *in vivo* mass excision of the barley cv Tweed cDNA library. All procedures were performed as described in Materials and Methods.

Marker lanes (M) containing  $\lambda$ HindIII DNA markers are shown and marker sizes are displayed on the left. Position of the pBluescript vector (2958bp) is shown on the right.



**Table 6.3:** Insert lengths in the barley cv Tweed cDNA library

Length of cDNA inserts from the plasmids of the twenty randomly-selected colonies described in Figure 6.4. Restriction fragment lengths were calculated using the shareware application program DNAfrag v3.03

Colony Number	Restriction Fragment Lengths (bp)	Total cDNA Insert Length (bp)
1	1016, 707, 436	2159
2	360	360
3	1107, 385	1482
4	742	742
5	1016, 707, 436	2159
6	1107	1107
7	973	973
8	1540	1540
9	1741	1741
10	548	548
11	891, 610	1501
12	1586	1586
13	926	926
14	1000	1000
15	1116	1116
16	823, 667	1490
17	2155	2155
18	1243	1243
19	727	727
20	555	555
Average		1255.5

### 6.2.2 Isolation of putative cDNA clones coding for nitrite reductase

Two probes were available for use in screening the barley cv Tweed cDNA library for nitrite reductase clones. The first, CIB808, was a 1850bp cDNA coding for maize nitrite reductase, including the whole of the coding region (Lahners *et al*, 1988). The second, BNiR1, was a 503bp cDNA coding for the 3' end of barley cv Maris Mink nitrite reductase, including 200bp of the coding region (Ward *et al*, 1995).

As the barley cv Tweed cDNA library was constructed using a combination of oligo-dT and random primers for first-strand cDNA synthesis, the library was expected to contain both oligo-dT-primed full-length nitrite reductase cDNA clones and shorter, overlapping random-primed nitrite reductase cDNA clones, covering various regions of the cDNA sequence. As barley contains a single copy of the nitrite reductase gene (J.L. Wray, unpublished), then overlapping hybridising clones would be expected to originate from the same gene. Therefore, it is possible to establish the complete nitrite reductase cDNA sequence by using either CIB808 or BNiR1 as a probe to isolate a full-length nitrite reductase cDNA clone, or by using CIB808 as a probe to isolate several overlapping cDNA clones which cover the entire nitrite reductase cDNA sequence. Several aliquots of the cDNA library were screened using both probes.



#### 6.2.2.1 Screening with CIB808

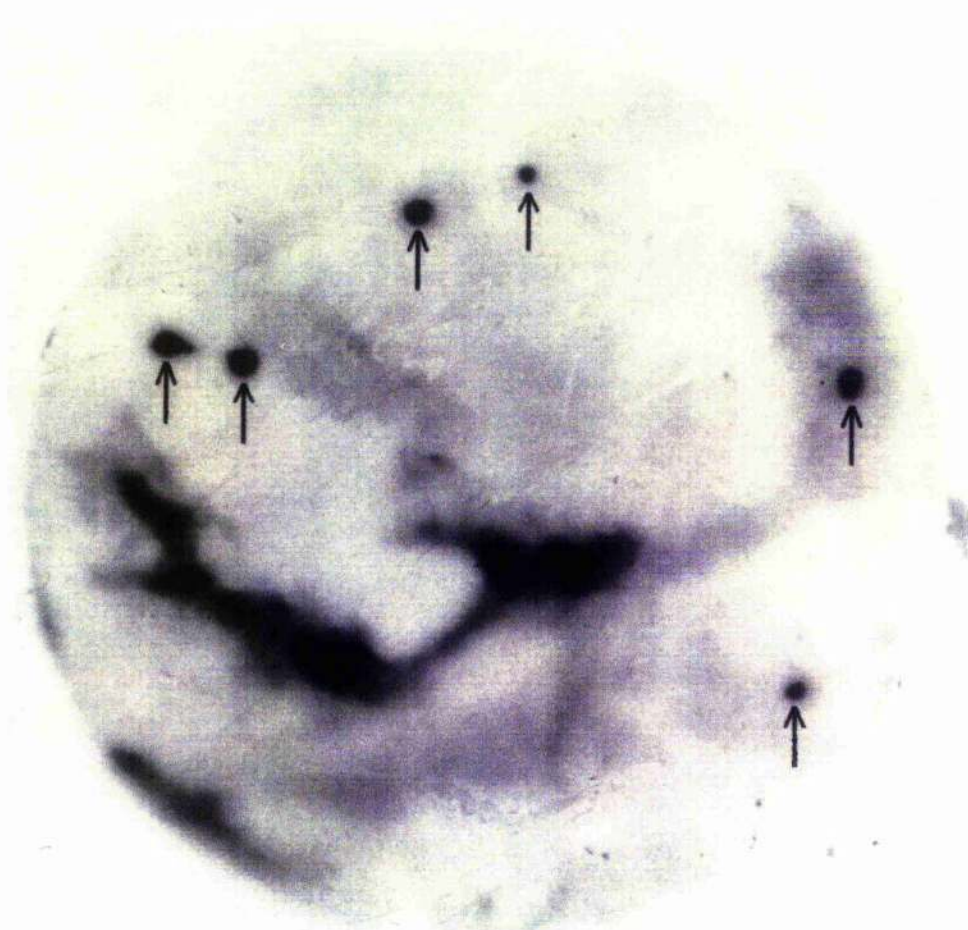
Approximately 150,000pfu from the cDNA library were screened using radiolabelled CIB808 under the conditions of low stringency required for heterologous probes as described in Materials and Methods, but unfortunately no positive clones were isolated.

#### 6.2.2.2 Screening with BNiR1

Approximately 150,000pfu were hybridised with radiolabelled BNiR1 under the conditions of high stringency required for homologous probes as described in Materials and Methods and seventeen duplicated positives were isolated from the first round of screening (Figure 6.5) and designated HvNiR1 to HvNiR17 (*Hordeum vulgare* Nitrite Reductase). These seventeen positives were then plated out at the dilutions which gave individual plaques and screened as before. Only twelve of the seventeen positives gave duplicated positives in this second round (Figure 6.6) and two or three plaques from each second round plate were isolated.

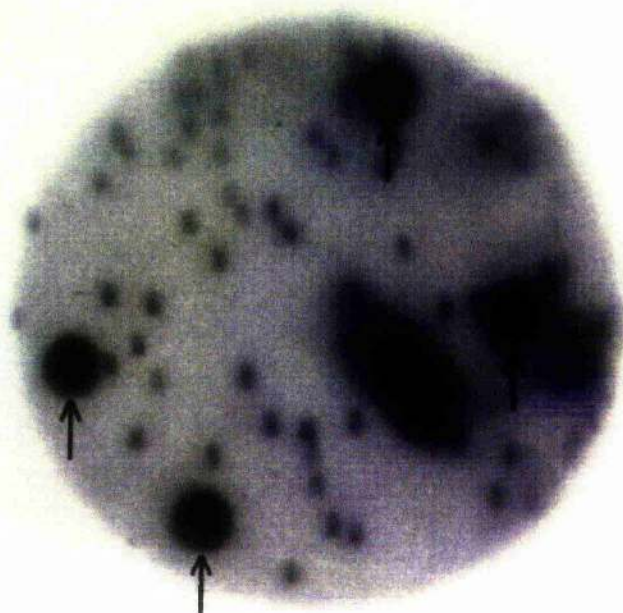
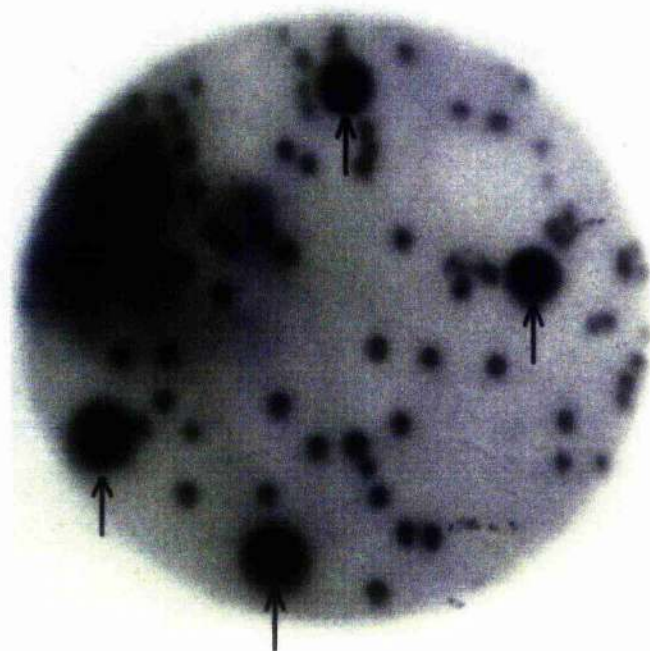
**Figure 6.5: First-round barley cDNA library screening with BNiR1**

An example of autoradiographs of duplicate first-round filters probed with radiolabelled BNiR1 cDNA and exposed to Kodak XAR™ film for 12-16 hours at -70°C. Filters were hybridised and washed according to the protocol for homologous probes described in Materials and Methods. Examples of duplicated plaques are indicated by arrows.



**Figure 6.6:** Second-round barley cDNA library screening with BNiR1

An example of autoradiographs of duplicate second-round filters probed with radiolabelled BNiR1 cDNA and exposed to Kodak XAR<sup>TM</sup> film for 12-16 hours at -70°C. Filters were hybridised and washed according to the protocol for homologous probes described in Materials and Methods. Examples of duplicated plaques are indicated by arrows.



### 6.2.2.3 Southern analysis of positive clones

PCR amplification of cDNA inserts from plaques using vector-specific primers and minigel analysis of the PCR products allows an early indication of clone sizes and subsequent Southern analysis of these gels can distinguish between true and false positives.

The cDNA inserts from two plaques isolated from the second-round screen of each of the twelve first-round true positive clones were amplified by PCR using primers designed from the flanking  $\lambda$ ZAPII M13 regions and subsequent minigel analyses (Figure 6.7a) showed PCR-amplified fragments with a size range of 655-2360bp, although the PCR products in some lanes were not visible. Subsequent hybridisation of Southern blots prepared from the minigels, using radiolabelled BNiR1 as a probe (Figure 6.7b), showed that the largest PCR-amplified fragment of 2360bp, isolated from HvNiR10, did not hybridise to BNiR1 and was either an artifact of the PCR reaction or was due to contamination of the plaques. The positive PCR-amplified fragment from this clone was 749bp in length, corresponding to a cDNA insert length of 568bp (Table 6.4). The largest clone, HvNiR6 (PCR-amplified fragment length 1524bp, cDNA insert length 1343bp; Table 6.4) was selected and plasmid was isolated from the  $\lambda$ ZAPII vector by *in vivo* excision. In order to verify the absence of phage contamination, the cDNA inserts from four resulting bacterial colonies were amplified by PCR. Minigel analysis (Figure 6.8) demonstrated that these PCR-amplified fragments were the same length as the PCR-amplified fragments from the HvNiR6 phage. It was concluded that the four bacterial colonies contained the cDNA HvNiR6 and glycerol stocks were prepared for these bacterial colonies. The plasmids within these colonies were designated pHvNiR61-pHvNiR64.



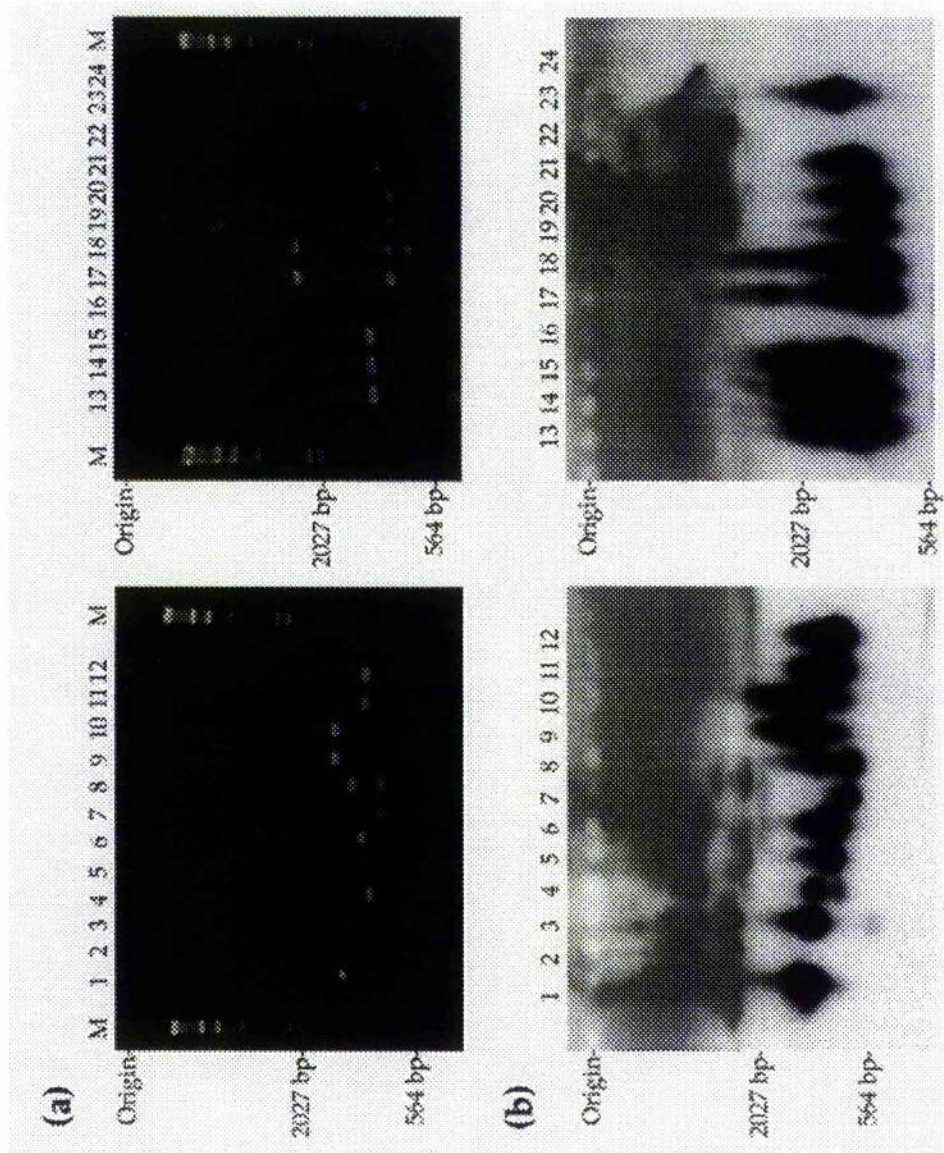
**Figure 6.7** Southern analysis of putative barley cv Tweed nitrite reductase cDNA clones

(a) Minigel analyses of the cDNA inserts amplified from twelve positive plaques by PCR, using primers designed from the flanking  $\lambda$ ZAPII M13 regions, and (b) Southern blots of (a) hybridised with radiolabelled BNiR1 and exposed to Kodak XAR™ film for 1 hour at -70°C.

PCR amplification, minigel analysis and Southern blotting/hybridisation were performed as described in Materials and Methods. Appropriate marker sizes are shown on the left.

Lanes: M - $\lambda$ HindIII markers			
1 - HvNiR1	5 - HvNiR3	9 - HvNiR6	13 - HvNiR8
2 - HvNiR1	6 - HvNiR3	10 - HvNiR6	14 - HvNiR8
3 - HvNiR2	7 - HvNiR4	11 - HvNiR7	15 - HvNiR9
4 - HvNiR2	8 - HvNiR4	12 - HvNiR7	16 - HvNiR9
			17 - HvNiR10
			18 - HvNiR10
			19 - HvNiR12
			20 - HvNiR12
			21 - HvNiR16
			22 - HvNiR16
			23 - HvNiR17
			24 - HvNiR17





**Table 6.4** Insert sizes of putative barley cv Tweed nitrite reductase cDNA clones

Putative Nitrite Reductase Clone	PCR-amplified fragment length (bp)	cDNA insert length (bp)
pHvNiR1	1262	1081
pHvNiR2	1144	963
pHvNiR3	930	749
pHvNiR4	655	474
pHvNiR6	1524	1343
pHvNiR7	833	652
pHvNiR8	1062	881
pHvNiR9	1062	881
pHvNiR10	749	568
pHvNiR12	749	568
pHvNiR16	821	640
pHvNiR17	1062	881
Average Insert Size		806.75

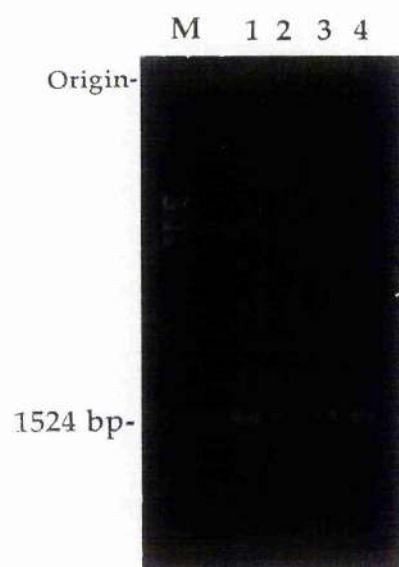
Insert sizes of the putative barley nitrite reductase cDNA clones described in Figure 6.7. PCR-amplified fragment lengths were calculated using the shareware application program DNAfrag v3.03. cDNA insert lengths were determined by subtracting the length of the flanking  $\lambda$ ZAPII M13 regions, which are also amplified by the M13 primers, from the PCR-amplified fragment length.

**Figure 6.8: Minigel analysis of the cDNA insert from HvNiR6**

Minigel analysis of cDNA inserts amplified by PCR from four bacterial colonies selected, after *in vivo* excision of the putative nitrite reductase cDNA clone HvNiR6, using primers designed from the flanking M13 regions.

PCR amplification of cDNA inserts from bacterial colonies and minigel analysis were performed as described in Materials and Methods. Band sizes are shown on the left.

Lanes: M,  $\lambda$ HindIII DNA markers; 1, pHvNiR61; 2, pHvNiR62; 3, pHvNiR63; 4, pHvNiR64.



### 6.2.3 Partial sequencing of clone HvNiR61

The putative barley cv Tweed nitrite reductase cDNA HvNiR61 was sequenced at the 5' and 3' ends using primers designed from the flanking M13 regions present in the pBluescript vector and the sequence was used to search the EMBL database. The 5' sequence, 68 bases within the coding region (Figure 6.9a), and the 3' sequence, 182 bases in the 3' untranslated region (Figure 6.9b), of HvNiR61 were submitted to the EMBL database and high homologies were found with known higher plant nitrite reductase nucleotide sequences (Table 6.5). The 5' sequence of HvNiR61 was 89% homologous to regions of both maize nitrite reductase and rice nitrite reductase nucleotide sequences. These regions of homology began 1273bp and 1135bp from the 5' ends of the rice and maize nitrite reductase clones respectively, which concurs with the estimation of HvNiR61 length as approximately 1.3kb (1000bp short of full length). The HvNiR61 3' sequence was 100% homologous to the 3' sequence of the barley cv Maris Mink nitrite reductase cDNA BNiR1, thus confirming the identity of HvNiR61 as a nitrite reductase cDNA clone.

Whilst the EMBL database search using the elucidated 5' sequence of HvNiR61 returned homologies only with the rice and maize nitrite reductase sequences, searches using the elucidated 3' sequence of HvNiR61 returned homologies with many higher plant sequences. This was ascribed to the presence of a poly-A<sup>+</sup> tail in the HvNiR61 sequence which shows high homology to the poly-A<sup>+</sup> tails of other unrelated higher plant nucleotide sequences so these homologies were discounted.

Elucidated 5' and 3' nucleotide sequence comparisons between HvNiR61 and higher plant nitrite reductase clones are shown in Figure 6.10.



(a)

CTGCAGGCCG	CTGGACATGT	TCGAGCTGGC	CCTGCCTCGC	CGACGAGTAC	50
TGGTCCTGGC	TGACGTCC				68

(b)

ATGGGTTGCG	GCTGATGTAA	AGTAGGCAAT	GCCCGCATGC	TTCCATGGCA	50
CACGCTTGTA	CCACGTTTTG	GCTCGTTGTC	GGAGCCCAA	GATTTTCGGT	100
TACGTTACAG	GGGATGATTG	AATTGATGAT	ATAAATAAAG	AAGCAGATTA	150
TATGTAGTTT	TTCAAAAAA	AAAAAAAAA	AA		182

**Figure 6.9** Partial nucleotide sequence of HvNiR61

Nucleotide sequence of the (a) 5' and (b) 3' ends of the cDNA insert from pHvNiR61. DNA sequencing was performed as described in Materials and Methods.

**Table 6.5** Sequence homology ratings for HvNiR61 at the nucleotide level

Comparison	Score	Identity	Homology
5' HvNiR61 v maize nitrite reductase	98	43/51	89%
5' HvNiR61 v rice nitrite reductase	97	41/48	89%
3' HvNiR61 v barley cv Maris Mink nitrite reductase	865	177/182	100%

Degrees of homology of the 5' and 3' nucleotide sequences from HvNiR61 (Figure 6.9) to the maize nitrite reductase, rice nitrite reductase and barley cv Maris Mink nitrite reductase nucleotide sequences (references given in Table 1.1; Chapter 1). Column 1 (Score) shows the overall homology rating proposed by the EMBL database search, column 2 (Identity) shows the number of matching nucleotides and column 3 (Homology) shows the overall percentage homology. This data was generated by EMBL database searches using the BLASTN program of the University of Wisconsin GCG v8.1-UNIX sequence analysis software package.



**Figure 6.10: Nucleotide sequence comparison of HvNiR61 and higher plant nitrite reductases**

Nucleotide sequence comparisons between the (a) 5' region of HvNiR61 and the maize nitrite reductase cDNA CIB808, (b) the 5' region of HvNiR61 and rice nitrite reductase cDNA and (c) the 3' region of HvNiR61 and the barley cv Maris Mink nitrite reductase cDNA BNiR1.

Sequences were aligned using the BESTFIT program from the University of Wisconsin GCG v8.1-UNIX sequence analysis software package and gaps (dots) were introduced where necessary to maximise homology. References are given in Table 1.1.



#### 6.2.4 Northern analysis of clone HvNiR61

A northern blot was prepared from total RNA extracted from the leaf tissue of 7 day-old, white-light-grown barley cv Tweed plants, either grown in the absence of nitrate or treated with 25mM nitrate for 18 hours in the light. The blot was hybridised with radiolabelled insert from pHvNiR61 and Figure 6.11 demonstrates that, as expected for a nitrite reductase gene, HvNiR61 expression is induced by nitrate. The size of the hybridising transcript is 2.3kb, the same size as *nii* transcript in barley leaf.

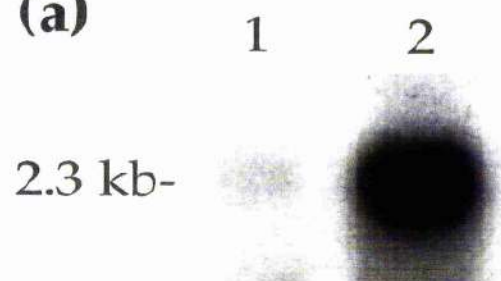
**Figure 6.11: Northern analysis of HvNiR61 transcript expression**

(a) Northern blot of (b) agarose gel showing total RNA extracted from leaf tissue of 7 day-old, green barley cv Tweed plants, hybridised with radiolabelled HvNiR61 and exposed to Kodak XAR™ film for 16 hours at -70°C.

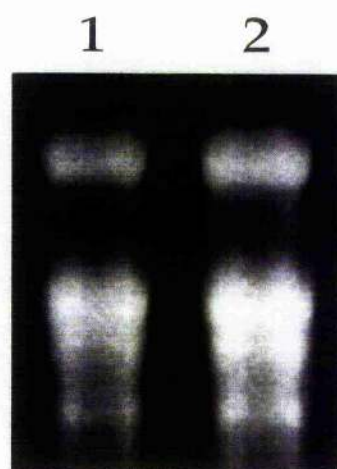
Lanes: 1, Total leaf RNA from plants grown in the absence of nitrate; 2, Total leaf RNA from plants treated with nitrate in the light for 18 hours.

Total RNA extraction, agarose gel electrophoresis and northern blotting and hybridisation were performed as described in Materials and Methods. Band sizes are shown on the left.

**(a)**



**(b)**



### 6.2.5 Nitrite reductase gene copy number in higher plants

As described previously, nitrite reductase (*Nii*) gene copy number differs between species. There has been little research into nitrite reduction in potato (*Solanum tuberosum*) and, although the the ancestral lineage of this species is unclear, *Solanum tuberosum* is a tetraploid species (Croy *et al*, 1993), therefore it is possible that this species has more than one copy of the *Nii* gene.

A *Solanum tuberosum* cv Desireé leaf cDNA library, constructed in  $\lambda$ ZAP, was available for study (Kossmann *et al*, 1992). Approximately 135,000pfu from this cDNA library were screened as described for the barley cv Tweed cDNA library, using radiolabelled insert from the heterologous tobacco leaf nitrite reductase cDNA *nir-3* (Kronenberger *et al*, 1993), which is expressed only in tobacco leaves. Eighteen positive clones, designated StNiR1-18 (*Solanum tuberosum* Nitrite Reductase) were isolated after one round of screening, thirteen of which produced duplicated positive plaques after a second round. The cDNA inserts from these thirteen putative potato nitrite reductase clones were isolated by PCR amplification using the flanking M13 regions of the  $\lambda$ ZAP vector and minigel analysis (Figure 6.12) shows several PCR-amplified fragments of approximately 2.3kb, which are probably full-length given that potato leaf nitrite reductase transcript is 2.1kb (N.Harris, University of St Andrews, UK; personal communication).

The two largest of these clones, StNiR12 and StNiR14, gave PCR-amplified fragment sizes of 2328bp and 2332bp respectively (corresponding to cDNA insert sizes of 2145bp and 2149bp, respectively) and their plasmids were isolated by *in vivo* excision. Sequencing of StNiR12 and StNiR14 was performed as for the barley nitrite reductase cDNA HvNiR61 and subsequent EMBL database searches (Table 6.6) revealed high homology of the 5' nucleotide sequences from both StNiR12 and StNiR14 to the same 5'

untranslated region of the tobacco nitrite reductase cDNA *nir-1* nucleotide sequence. Elucidated 5' nucleotide sequence comparisons of StNiR12 and StNiR14 to the tobacco nitrite reductase cDNA *nir-1* are shown in Figure 6.13. There were, however, differences between the two potato nitrite reductase cDNA 5' sequences (Figure 6.13), suggesting that at least two different nitrite reductase genes are expressed in potato leaf. Subsequent studies of potato leaf have demonstrated that StNiR12 hybridises to a nitrate-induced transcript of 2.1kb, the same size as the nitrite reductase transcript (N. Harris, University of St Andrews, UK; personal communication).

Sequencing further into the 5' region of StNiR12 and StNiR14 will be necessary to provide an overlap with the two other characterised tobacco nitrite reductase cDNAs, *nir-2* and *nir-3*, hence it may be that StNiR12 and/or StNiR14 possess greater homology to one or both of these cDNAs than to the tobacco *nir-1* clone.

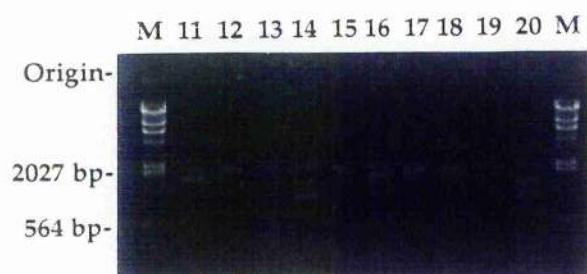
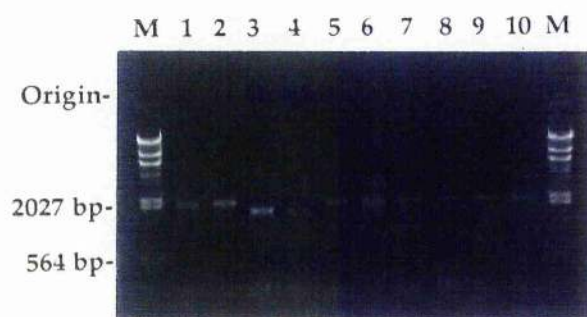


**Figure 6.12** Minigel analysis of PCR amplified fragments from putative potato nitrite reductase cDNA clones

Minigel analysis of cDNA inserts from thirteen positive plaques, amplified by PCR, using primers designed from the flanking  $\lambda$ ZAPII M13 regions.

PCR amplification and minigel analysis were performed as described in Materials and Methods. Appropriate marker sizes are shown on the left.

Lanes:	M - $\lambda$ HindIII markers	
	1 - StNiR3	11 - StNiR13
	2 - StNiR5	12 - StNiR13
	3 - StNiR6	13 - StNiR14
	4 - StNiR6	14 - StNiR14
	5 - StNiR7	15 - StNiR15
	6 - StNiR9	16 - StNiR15
	7 - StNiR10	17 - StNiR16
	8 - StNiR11	18 - StNiR16
	9 - StNiR12	19 - StNiR17
	10 - StNiR12	20 - StNiR17



**Table 6.6** Sequence homology ratings for StNiR12 and StNiR14 at the nucleotide level

Comparison	Score	Identity	Homology
5' StNiR12 v Tobacco <i>nir-1</i> nitrite reductase	244	81/88	86%
5' StNiR14 v Tobacco <i>nir-1</i> nitrite reductase	170	89/123	73%

Degrees of homology of the 5' nucleotide sequences from StNiR12 and StNiR14 to the tobacco nitrite reductase cDNA *nir-1* nucleotide sequence (Vaucheret *et al*, 1992). Column 1 (Score) shows the overall homology rating proposed by the EMBL database search, column 2 (Identity) shows the number of matching nucleotides and column 3 (Homology) shows the overall percentage homology. This data was generated by EMBL database searches using the BLASTN program of the University of Wisconsin GCG v8.1-UNIX sequence analysis software package.

**Figure 6.13: Comparison of StNiR12 and StNiR14 nucleotide sequences**

Nucleotide sequence comparison of (a) the 5' region of StNiR12 and the tobacco nitrite reductase cDNA *nir-1*, (b) the 5' region of StNiR14 and the tobacco nitrite reductase cDNA *nir-1* and (c) the 5' region of StNiR12 and the 5' region of StNiR 14.

Sequences were aligned using the BESTFIT program from the University of Wisconsin GCG v8.1-UNIX sequence analysis software package and gaps (dots) were introduced where necessary to maximise homology.

(a)

```
5' StNiR12 66 TTTCTATCAAATTTTGGCACCTTCATTGCCAAATCCAACCTAGATTTTCC 115
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Tob nir-1 1 TTTCTATTAAATTTCTGGCACCTTCATTGCCAAATCCAGCTAGATTTTCC 50

5' StNiR12 116 AAGAGTAGTATTGTCAAGCTCAATGCAACTCCCTGACGCAGCAAGTGGCT 165
||| || ||||| ||||| ||||| ||| || |||||
Tob nir-1 51 AAG...AATGCTGTCAAGCTCCACGCAACTC....CGCCGCTGTGGCA 92

5' StNiR12 166 GC.....GTGGGGACTCCAGAGGTTGCTGCTGAGAGACTAGA 202
|| || || ||||| ||||| ||||| |||||
Tob nir-1 93 GCGCCGCCAGCTGGTGCTCCAGAGGTTGCTGCTGAGAGGCTAGA 136
```

(b)

```
5' StNiR14 63 TCTCGGTAAATTTTCAGCTACTTCACTTCCAAATTC TAATAGATTTTC. 111
|| ||||| || ||||| ||||| || ||||| |||||
Tob nir-1 1 TTTCTATTAAATTTCTGGCACCTTCATTGCCAAATCCAGCTAGATTTTCC 50

5' StNiR14 112 .....CAAGCTTCATGCTACTCCACCGCAGACCGGTGCGGATCC 150
||||| || || ||| ||||| || || || |||
Tob nir-1 51 AAGAATGCTGTCAAGCTCCACGCAACT...CCGCCGCTGTGGCAGCGCC 97

5' StNiR14 151 GTCATCTGGGGCGGCGGAGATAGCTGCTGAGAGACTAGAGCCTAGAGT.. 198
| || |||| || || || ||||| ||||| || |||||
Tob nir-1 98 GCCAGCTGGTGCTCCAGAGGTTGCTGCTGAGAGGCTAGAACCCAGAGTTG 147

5' StNiR14 199 GAGCAAAAGATGGGTA 214
| ||||| || ||
Tob nir-1 148 AGGAAAAAGATGGTTA 163
```

(c)

```
5' StNiR12 52 CAAATGGCATCTTTTCTATCAAATTTTGGCACCTTCATTGCCAAATC 101
||||| ||||| || ||||| || ||||| |||||
5' StNiR14 49 CGAAATGACATCTTTCTCGGTAAATTTTCAGCTACTTCACTTCCAAATT 98

5' StNiR12 102 CAACCTAGATTTTCCAAGAGTAGTATTGTCAAGCTCAATGCAACTCC.CTG 150
|| ||||| || ||||| ||||| ||||| |||||
5' StNiR14 99 CTAATAGATTTTC.....CAAGCTTCATGCTACTCCACCG 133

5' StNiR12 151 ACGCAGCAAGTGGTGCGT....GGGGACTCCAGAGGTTGCTGCTGAGAG 196
| || || || ||||| || || || ||||| |||||
5' StNiR14 134 CAGACCGGTGCGGATCCGTCATCTGGGGCGGCGGAGATAGCTGCTGAGAG 183

5' StNiR12 197 ACTAGA 202
|||||
5' StNiR14 184 ACTAGA 189
```

## 6.3 DISCUSSION

### 6.3.1 Construction of the barley cv Tweed cDNA library

Attempts to isolate a full-length nitrite reductase cDNA clone from the barley cv Tweed cDNA library were unsuccessful, and this is most likely due to a poor quality cDNA library. The most common reason for this is a poor quality poly A<sup>+</sup> RNA template (Sambrook *et al*, 1989). In these studies, however this does not appear to be the case. Both total RNA and poly A<sup>+</sup> RNA samples displayed OD<sub>260</sub>/OD<sub>280</sub> ratios (Table 6.2) within the acceptable range of 1.8-2.0 (from manufacturers instructions for Oligotex-dT kit, QIAGEN, UK). There did not appear to be a problem with RNA integrity (Figure 6.2) and poly A<sup>+</sup> RNA of up to 6kb was present. However the cDNA library constructed from this poly A<sup>+</sup> RNA contained an average insert size of less than 1300bp (Table 6.3) even after size-fractionation of the library to >400bp and this suggests that the poly A<sup>+</sup> RNA may have become degraded in transit or during first-strand cDNA synthesis.

### 6.3.2 Isolation of HvNiR61

Attempts to isolate barley nitrite reductase cDNA clones using the maize nitrite reductase cDNA clone CIB808 as a heterologous probe were unsuccessful, probably due to the hybridisation conditions used although these same conditions allowed the isolation of thirteen positive potato cDNA clones using the tobacco nitrite reductase cDNA clone *nir-3* as a heterologous probe (Figure 6.12). Twelve positive barley cDNA clones were, however, isolated using the homologous probe BNiR1 after two rounds of screening and these were studied further.

PCR-amplification and subsequent Southern analysis of these putative barley nitrite reductase cDNA clones (Figure 6.7) showed the largest putative nitrite reductase (HvNiR61) to be 1343bp in length and the smallest to be 474bp in length (Table 6.4). The PCR-amplified fragment lengths for these clones (1524bp and 655bp, respectively) are longer as they include the  $\lambda$ ZAPII regions containing the M13 primers. The failure to isolate larger clones is, as has been discussed, probably due to the poor quality of the cDNA library as thirteen potato nitrite reductase cDNA clones were isolated from the potato cv Desiree leaf cDNA library using essentially the same screening conditions (Figure 6.12).

### 6.3.3 Sequence of HvNiR61

The 5' and 3' partial nucleotide sequences of the barley cDNA clone HvNiR61 identified this clone as a nitrite reductase apoprotein cDNA, with high homology shown to maize (89%), rice (89%) and barley cv Maris Mink (100%) nitrite reductase cDNA nucleotide sequences (References given in Table 1.1). The positions of homology of the 5' HvNiR61 nucleotide sequence to the maize and rice nitrite reductase cDNA sequences were 1135bp and 1273bp from the 5' end of the maize and rice clones respectively and concurred with the estimation of HvNiR61 length as 1343 bp (approximately 950bp shorter than the barley *nii* transcript). It is of interest that the 3' nucleotide sequence of the barley cv Maris Mink cDNA possessed a group of five nucleotides that were not present in the HvNiR61 sequence (Figure 6.10) and a gap had to be added by the BESTFIT sequence comparison program (Figure 6.10) to give a 100% homology rating between these two barley nitrite reductase cDNA clones (Figure 6.10). This difference is unlikely to be due to sequencing error as five nucleotides are involved and suggest that the



untranslated 3' regions of the nitrite reductase apoprotein gene are not conserved among barley cultivars.

#### 6.3.4 Northern analysis of HvNiR61

As expected for a nitrite reductase cDNA clone, HvNiR61 hybridises to a 2.3kb nitrate induced transcript present in the leaf of barley, which provides further evidence that HvNiR61 encodes the nitrite reductase apoprotein gene from barley (Figure 6.11).

As a full-length nitrite reductase cDNA clone from barley cv Tweed was necessary for the further study of the *nir1* mutants, the barley nitrite reductase cDNA clone HvNiR61 was not characterised any further.

#### 6.3.5 Isolation of potato nitrite reductase cDNA clones

Thirteen hybridising clones were isolated from the potato cv Désirée λZAP leaf cDNA library (Kossmann *et al*, 1992) when the heterologous tobacco leaf nitrite reductase cDNA clone *nir-3* (Kronenberger *et al*, 1993) as a probe (Figure 6.12). Homologies of the 5' partial nucleotide sequences of the two largest clones StNiR12 and StNiR14 to the tobacco leaf nitrite reductase cDNA clone *nir-1* were 86% and 73% respectively and subsequent studies have shown that one of these clones, StNiR12, hybridises to a nitrate-induced transcript of 2.1kb in potato leaf (N. Harris, University of St Andrews, UK; personal communication), the same size as potato leaf nitrite reductase transcript. StNiR14 was not used in this study (N. Harris, University of St Andrews, UK; personal communication). However, comparison of the 5' partial nucleotide sequence between StNiR12 and StNiR14 show that the untranslated regions, at least, of these clones are different (Figure 6.13) and

this suggests that potato leaf expresses at least two different nitrite reductase genes.

## CHAPTER 7

### Discussion

## 7.1 Growth characteristics and maintenance of nitrite-accumulating selections

Selected nitrite accumulators could not be maintained on nitrate since it is likely that nitrogen would not be assimilated into amino-nitrogen due to the block in the pathway. Thus, the plants would suffer from nitrogen starvation while nitrite may continue to accumulate to toxic levels, either event proving lethal to the plant. The effect of nitrate on the growth of nitrite-accumulating selections was demonstrated when the nitrite-accumulating selections STA1010, STA2760 and STA4169 were transferred from nitrate-free vermiculite to compost. Leaves withered from the tip downwards and within 12 (STA1010 and STA2760) or 14 (STA4169) days the plants had died. The nitrite-accumulating selection STA3999 also dies within 12 days of transfer to compost (Duncanson *et al*, 1993).

Nitrite-accumulating selections can be germinated and grown on nitrate-free vermiculite and maintained to flowering after transfer to hydroponic culture using 1mM glutamine as the sole nitrogen source (Duncanson *et al*, 1993). However, the selections maintained using this method displayed abnormal growth characteristics (weak and slow growth, and poor seed setting) or failed to survive (Duncanson, 1990).

The nitrite-accumulating selections used for cross-pollination in this work were maintained in hydroponic culture using 2mM ammonium chloride as the sole nitrogen source, and although growth of these selections in hydroponic culture was generally slower than that of nitrite non-accumulating plants grown in compost, all hydroponically-grown selections survived to flowering. The major problem encountered here was the microbial contamination of the hydroponic medium, although the risk of contamination was reduced by using fresh medium every two days and

contamination of the medium was less of a problem when the selections were established with a strong root system.

## 7.2 Genetic analysis of nitrite-accumulating selections

Back-crosses of the nitrite-accumulating selections STA1010, STA2760 and STA4169 to the wild-type cultivars Klaxon, Klaxon and Golden Promise respectively were performed successfully and self-pollination of F<sub>1</sub> seed from these crosses produced F<sub>2</sub> populations which segregated in a ratio of 3:1 nitrite non-accumulators:nitrite accumulators. These data demonstrates that the mutations responsible for *in vitro* leaf nitrite accumulation in the selections STA1010, STA2760 and STA4169 are recessive mutations within single nuclear loci. The nitrite-accumulating selection STA3999 also carries a recessive mutation in a single nuclear locus, which has been designated *Nir1* (Duncanson *et al*, 1993).

Allelism tests have shown that the mutation carried by STA4169 is allelic to the mutations carried by STA1010 and STA2760. Other studies (J.L. Wray, unpublished) have shown that the mutation carried by STA3999 is allelic to the mutation carried by STA1010, hence all four selections are defective at the *Nir1* locus. Since the selections STA1010 and STA2760 are derived from the same mutagenised population of cv Klaxon seed (J.L. Wray, University of St Andrews, UK; personal communication) the possibility that they are derived from the same mutational event cannot be discounted. Thus, at least three, and perhaps four, independent mutations at the *Nir1* locus have been isolated.

### 7.3 Biochemical characterisation of the *nir1* mutants

#### 7.3.1 *In vivo* leaf nitrite accumulation

*In vivo* nitrite accumulation in the leaves of the selections STA1010, STA2760 and STA4169 occurs within 2 hours of exposure to 50mM potassium nitrate. Accumulation of nitrite continues up to at least 18 hours in these selections after exposure to nitrate. Leaf nitrite levels of between 900nmoles nitrite/g fresh weight and 988nmoles nitrite/g fresh weight are observed in M and F populations of the selections STA1010 and STA2760 after treatment with nitrate for 18 hours. These levels are slightly lower than the level found in the leaf tissue of the selection STA3999 under the same conditions (approximately 1300nmoles nitrite/g fresh weight) (Duncanson *et al*, 1993). Leaf nitrite levels observed within M and F populations of the selection STA4169 after 18 hours treatment with nitrate were much lower (570nmoles nitrite/g fresh weight to 620nmoles/g fresh weight) than the levels found in the three other selections. Lower levels of *in vivo* nitrite accumulation in STA4169, in comparison with the selections STA1010, STA2760 and STA3999, would explain the longer survival time of the selection STA4169 in compost, if death in compost is due to the accumulation of nitrite to toxic levels.

Studies performed by Vaucheret *et al* (1992a) have demonstrated a similar phenotype in transgenic *Nicotiana tabacum* expressing a tobacco leaf *Nii* antisense construct, which also accumulates nitrite in the leaf to five times the level found in wild-type plants.

### 7.3.2 Regulation of nitrite reductase gene expression by nitrate

Biochemical studies have been performed using plants within the F<sub>2</sub> populations derived from the crosses STA1010 × Klaxon, STA2760 × Klaxon and STA4169 × Golden Promise.

Like the previously characterised *nir1* mutant STA3999, the *nir1* mutants STA1010, STA2760 and STA4169 lack the detectable nitrite reductase cross-reacting material in leaf and root which is present in the leaf and root of wild-type plants and non-mutant siblings. This suggests that a single gene is responsible for the presence of nitrite reductase protein in both the leaf and root of barley.

*In vitro* methyl viologen nitrite reductase activity assays show that the four *nir1* mutants possess greatly reduced nitrite reductase activity in the leaf, being 5-8% of leaf nitrite reductase activity in wild-type and non-mutant sibling plants after treatment with nitrate in the light. This reduced level of leaf nitrite reductase activity is also present in plants not treated with nitrate. Therefore, the low nitrite reductase activity in *nir1* mutants is not nitrate-inducible, suggesting that the *Nir1* locus is responsible for the "basal" as well as "inducible" levels of nitrite reductase activity found in the leaf of wild-type barley plants (Duncanson *et al*, 1992).

This low level nitrite reduction in the leaf of *nir1* mutants is unlikely to be due to functional nitrite reductase enzyme as there is no detectable nitrite reductase cross-reacting material in *nir1* mutant leaf tissue. It is possible that nitrite may be metabolised by the action of sulphite reductase which, like nitrite reductase, contains siroheme and an Fe<sub>4</sub>S<sub>4</sub> centre and is able to catalyse the ferredoxin or methyl viologen-dependent six-electron reduction of sulphite to sulphide and nitrite to ammonium ions in spinach (Krueger and Siegel, 1982). The *nir1* mutant STA3999 possesses functional



sulphite reductase (Gilkes, 1994), which also suggests that the *Nir1* locus is not involved in prosthetic group biosynthesis.

The observed *in vitro* leaf nitrite reductase activity may also be due to a purely chemical reaction. Nitrite is present in *in vitro* nitrite reductase assays at an initial concentration of 2mM and the methyl viologen present in the test assay may react with components of the leaf tissue extract and in some way cause a decrease in the level of nitrite in comparison to the controls, which do not contain methyl viologen.

Lastly, nitrite metabolism may occur through the action of some, as yet unidentified, biological activity. Such an activity would not necessarily be primarily involved with nitrite metabolism, but should be able to convert nitrite into some other compound in selected lines where there is no competition for nitrite by nitrite reductase.

Northern analysis of leaf nitrite reductase apoprotein (*nii*) transcript demonstrates that *nir1* mutants produce leaf *nii* transcript of wild-type size (2.3kb) and at approximately wild-type levels. This suggests that the *nir1* phenotype is not due to a defect in a regulatory locus as such a defect would be expected to reduce or abolish synthesis of *nii* transcript.

### 7.3.3 Regulation of nitrate reductase gene expression by nitrate

Regulation of nitrate reductase in the *nir1* mutants is less clear. *In vitro* leaf NADH-nitrate reductase activity is over two (STA1010, STA2760 and STA3999) or one-and-a-half (STA4169) times higher in the *nir1* mutants as compared to wild-type plants after nitrate treatment for 18 hours, suggesting that the lack of an end product to the nitrate assimilation pathway, probably glutamine, is causing overexpression of the *nar1* gene in the *nir1* mutants. The lower nitrate reductase activities seen in STA4169, as compared to the three other *nir1* mutants, concurs with the observations of lower *in vivo* leaf

nitrite levels after nitrate treatment and longer survival time in compost of the *nir1* mutant STA4169, as lower leaf nitrate reductase levels will lead to lower levels of leaf nitrite accumulation.

However, leaf NADH-nitrate reductase (*nar1*) transcript levels in the *nir1* mutants are not overexpressed after nitrate treatment for 18 hours. The buffer used to extract barley leaf tissue for *in vitro* enzyme assays (Kuo *et al*, 1980) contains EDTA which chelates magnesium in the tissue extract and thus abolishes the effect of post-translational inactivation of the nitrate reductase apoenzyme (Chapter 1). Hence, the higher *in vitro* leaf NADH-nitrate reductase activities observed in the *nir1* mutants are not due to the ratio of inactivated NR protein:activated NR protein being lower in the *nir1* mutants than in wild-type plants. It is possible, however, that the *nir1* mutants have lower *in vivo* nitrate reductase protein degradation rates than are present in wild-type plants, which would cause an accumulation of nitrate reductase protein. This would be expected to lead to higher leaf nitrate reductase activities in the *nir1* mutants even when *nar1* transcript is at wild-type levels.

An interesting observation in this study was that *in vitro* leaf nitrate reductase activity levels in the *nir1* mutants are approximately four (STA1010, STA2760 and STA3999) or two (STA4169) times higher than wild-type levels in plants not exposed to nitrate. Northern analysis has demonstrated that leaf *nar1* transcript is also overexpressed in the *nir1* mutants STA1010, STA2760 and STA3999 under the same conditions, although any increase of the leaf *nar1* transcript level in the *nir1* mutant STA4169 as compared to the wild-type level is undetectable.

One explanation for overexpression of *nar1* transcript and activity in the absence of nitrate is that the absence of nitrite reductase enzyme molecules alone is sufficient to cause overexpression of nitrate reductase, although this has no precedent in previous studies. While the nitrate

reductase enzyme in *Aspergillus nidulans* autogeneously regulates its own expression (Cove and Pateman, 1969), this effect has not been found in higher plants. Studies of *Nicotiana plumbaginifolia* have demonstrated that nitrate enhances the level of nitrate reductase transcript in a whole range of nitrate reductase-deficient *nir* mutants affected in different domains of the nitrate reductase apoprotein (Pouteau *et al*, 1989). This conserved nitrate-inducibility argues against a direct role of the nitrate reductase enzyme in its regulation by nitrate in higher plants so it is unlikely that the nitrite reductase enzyme has such a role in the regulation of nitrate reductase. The most likely explanation for *nar1* overexpression in selections STA1010, STA2760 and STA3999 not exposed to exogenous nitrate is that undetectable amounts of nitrate are present in the leaf tissue, either from seed reserves or from exogenous sources, and these low levels of nitrate induce the overexpression of leaf nitrate reductase activity and transcript in the *nir1* mutants more efficiently than the high levels of nitrate observed in the leaf tissue of *nir1* mutants treated with 25mM nitrate. This increased efficiency of leaf *nar1* induction at lower leaf nitrate levels has been demonstrated in timecourse studies of nitrate regulation of leaf *nar1* transcript in the *nir1* mutant STA3999. *In vivo* leaf nitrate levels up to 5.2 $\mu$ moles nitrate/g fresh weight can lead to overexpression of leaf *nar1* transcript in STA3999, a level which occurs in the leaf after 8 hours of exposure to 25mM nitrate. However, at higher leaf nitrate levels (up to 11.3 $\mu$ moles nitrate/g fresh weight) no overexpression of the leaf *nar1* transcript is observed. This data suggests that overexpression of leaf *nar1* transcript in STA3999 occurs at low leaf nitrate concentrations and causes an accumulation of leaf NADH-nitrate reductase enzyme molecules. This accumulated enzyme may possess a longer half-life than leaf NADH-nitrate reductase in the wild-type and subsequently lead to increased leaf NADH-nitrate reductase activity levels in STA3999 at high leaf

nitrate concentrations as compared to the wild-type, even though leaf *nar1* transcript is present at wild-type levels.

The *nir1* mutant STA4169 does not appear to overexpress leaf *nar1* transcript in the absence of nitrate. As STA4169 plants were germinated in the same seed tray as other *nir1* mutants, it can be assumed that the lack of overexpression of leaf *nar1* transcript in STA4169 is not due to a difference in growth conditions between STA4169 and the other selections. However, increased leaf NADH-nitrate reductase activity in nitrate-free plants of STA4169 suggests that some up-regulation of NADH-nitrate reductase is effective. This may be due to the accumulation of "basal" level NADH-nitrate reductase enzyme in the leaf tissue of STA4169. Nevertheless, it appears that regulation of NADH-nitrate reductase differs between the *nir1* mutant STA4169 and the *nir1* mutants STA1010, STA2760 and STA3999.

*In vitro* NADPH-nitrate reductase activity assays have shown that leaf NAD(P)H-nitrate reductase makes no contribution to these increases in leaf nitrate reductase activity in the *nir1* mutants.

Regulation of nitrate reductase gene expression in the *nir1* mutants shows similarities to the regulation of nitrate reductase gene expression in other nitrate assimilation mutants. Studies show that, like the barley *nir1* mutants, many *nia* and *cnx* mutants, where nitrate reductase activity is low or absent, overexpress nitrate reductase apoprotein mRNA under certain growth conditions (Kleinhofs *et al*, 1989; Pouteau *et al*, 1989; Wilkinson and Crawford 1991; Labrie *et al*, 1992). Overexpression of nitrate reductase does not appear to be detrimental in higher plants as studies in *Nicotiana plumbaginifolia* (Vincentz and Caboche, 1991) have demonstrated that constitutive expression of nitrate reductase allows normal growth and development of the plant.

Furthermore, studies using transgenic *Nicotiana tabacum*, which expresses a tobacco leaf *Nii* antisense construct (Vaucheret *et al*, 1992a), show

similar phenotypic characteristics to the barley *nir1* mutants with regard to nitrate reductase regulation. Both the *nir1* mutants and the transgenic *Nicotiana* overexpress nitrate reductase activity and transcript under certain growth conditions, providing further support for the contention that the nitrate assimilation pathway is down-regulated by an end-product of nitrate assimilation, and possess nitrate reductase transcript which retains nitrate-inducibility.

These biochemical studies suggest that the *nir1* mutants STA1010, STA2760 and STA3999 possess similar, if not identical, phenotypes. However, biochemical analysis of the *nir1* mutant STA4169 demonstrate that STA4169 possesses a lower NADH-nitrate reductase activity than the three other selections and as a consequence nitrite does not accumulate in the leaf of STA4169 as rapidly as in the leaves of STA1010, STA2760 and STA3999 after nitrate treatment in the light. It therefore seems likely that STA4169 carries a mutation in the *Nir1* locus which is different to the mutations carried by the three other selections STA1010, STA2760 and STA3999 and this difference is responsible for the variations observed between the phenotypes. This, however, does not preclude the possibility that the mutations carried between the selections STA1010, STA2760 and STA4169 are also different.

#### 7.4 RFLP mapping of the *Nir1* locus

As *nir1* mutants produce *nii* transcript of wild-type size (2.3kb) and at wild-type levels in response to nitrate treatment, it is unlikely that the *nir1* mutation is present within a regulatory locus, as such a defect would be expected to reduce or prevent the synthesis of *nii* transcript.

An RFLP between the barley wild-type cultivars Tweed (major hybridising band at 11.5kb) and Golden Promise (major hybridising band at

7.5kb) when *Dra*I-digested DNA is probed using radiolabelled insert from the partial barley nitrite reductase cDNA clone pBNiR1 (Ward *et al*, 1995) has been demonstrated. An RFLP was not detected between the wild-type cv Tweed and the Tweed *nir1* mutant STA3999.

Cross-pollination of STA3999 to the wild-type cultivar Golden Promise has allowed the isolation of an F<sub>2</sub> population cosegregating for the Tweed RFLP and the *nir1* phenotype. Study of this F<sub>2</sub> population has allowed the identification of 84 F<sub>2</sub> plants which strictly cosegregate for the Tweed RFLP with the *nir1* phenotype from a total population of 312 F<sub>2</sub> plants. This shows no significant difference at the 5% level to the Mendelian 3:1 segregation ratio for a recessive nuclear mutation. Study of F<sub>2</sub> individuals and batches of F<sub>2</sub> nitrite accumulating plants found no evidence of recombination between the *nir1* phenotype and the heterozygous/Golden Promise RFLP. Study of 36 F<sub>2</sub> individuals found no recombination between the Tweed RFLP band at 11.5kb and the wild-type phenotype and it was assumed that none existed in the total nitrite non-accumulating sample of 228 nitrite non-accumulating F<sub>2</sub> plants.

By postulating that one of the 312 F<sub>2</sub> plants was recombinant, it was possible to map the *Nir1* locus to within 0.3cM of the *Nii* gene (W.T.B. Thomas, SCRI, Invergowrie, UK, personal communication).

## 7.5 Identity of the *Nir1* locus

At the outset of this work, it was postulated that mutations affecting a number of loci would be expected to produce plants which accumulate nitrite in the leaf after treatment with nitrate, such as mutations causing defects in the nitrite reductase apoprotein, in components of the chloroplast envelope, in stromal proteinases, in prosthetic group synthesis, in nitrite transport into the chloroplast (if this mechanism is protein mediated), in



electron donation to nitrite reductase or in the signal transduction pathway through which light, nitrate and the plastidic factor operate to regulate the synthesis of nitrite reductase. However, mutations in some of these components of nitrite reduction would be lethal and unselectable.

These studies have shown that the *Nir1* locus is responsible for the formation of nitrite reductase protein in both the leaf and root of barley, and for the "basal" and "inducible" levels of nitrite reductase activity found in the wild-type. The *nir1* mutants produce nitrate-inducible *nii* transcript of wild-type size (2.3kb) and at approximately wild-type levels suggesting that the *Nir1* locus does not encode a regulatory component of nitrite reduction. RFLP mapping of the *Nir1* locus to the nitrite reductase apoprotein gene *Nii* demonstrated that these two loci are tightly-linked, and as no recombination was found between the two loci it is more likely that they are allelic.

If this is the case, there are several possibilities as to the location of the *nir1* mutation within the *Nii* gene. Mutations affecting either the translation of the *nii* transcript or the stability of the nitrite reductase protein would be expected to produce the *nir1* phenotype.

Translation of the *nii* transcript could be affected by mutations in ribosomal binding regions of the transcript, mutations producing a stop codon at the 5' end of the *nii* coding region or mutations altering the start codon within the *nii* transcript. Stability of the nitrite reductase protein may be affected by mutations in *Nii* regions encoding the prosthetic group binding sites of the nitrite reductase protein or mutations in the *Nii* coding region affecting the structure of the nitrite reductase protein.

Studies to confirm the identity of the *Nir1* locus as *Nii* and to determine the intragenic location of the *nir1* mutation if present by establishing the full-length *Nii* cDNA sequences for the Tweed *nir1* mutant STA3999 and the wild-type cv Tweed for comparative purposes were



unsuccessful as attempts to isolate an *Nii* cDNA clone from a barley cv Tweed cDNA library yielded only partial *Nii* clones.

## 7.6 Isolation of a partial barley nitrite reductase cDNA clone

A barley cv Tweed cDNA library has been constructed using poly A<sup>+</sup> RNA isolated from both leaf and root tissue. Screening of this library using the maize nitrite reductase cDNA clone CIB808 (Lahners *et al*, 1988) as a probe failed to isolate any hybridising plaques. This may be because the conditions used for colony hybridisation and washing (55°C, filters washed to 0.2 x SSPE/0.1% SDS) were too stringent to allow hybridisation of this heterologous probe, although these hybridising and washing conditions were used successfully for the isolation of thirteen hybridising clones from a potato cv Desireé leaf cDNA library, using the tobacco leaf nitrite reductase cDNA clone *nir-3* (Kronenberger, 1993) as a heterologous probe.

However, in a parallel study seventeen hybridising clones were isolated from the barley cv Tweed cDNA library after one round of screening using the partial barley nitrite reductase cDNA clone BNiR1 (Ward *et al*, 1995) as a homologous probe. Five of these seventeen clones did not produce hybridising plaques in a second round of screening under identical conditions. The fact that a positive signal was initially obtained suggests that non-specific hybridisation had occurred, possibly due to some interaction between the probe used, BNiR1 (Ward *et al*, 1995), and the  $\lambda$ ZAPII vector used for cDNA library construction (Stratagene, UK). In addition, if a minority of plaques formed are larger than the majority, an enhanced background signal can be obtained making these larger plaques appear to give a more intense hybridisation signal and cause them to stand out as positive clones.

The cDNA inserts from the remaining twelve hybridising clones were amplified by PCR using primers designed from the  $\lambda$ ZAPII M13 regions flanking the 5' and 3' ends of the cDNA inserts. Minigel analysis and subsequent Southern analysis revealed an average putative nitrite reductase cDNA clone size of 807bp, some 1500bp shorter than the expected full length of 2300bp, and the largest nitrite reductase cDNA was 1343bp, approximately 950bp shorter than the expected full length of 2300bp. This suggests that the cDNA library is of a poor quality, containing only partial cDNA clones of a very short length. Analysis of randomly-selected cDNA clones estimated the average cDNA insert size of the library as 1256bp and as the cDNA inserts for the library had been size fractionated to >400bp it is unlikely that the library contains many cDNA inserts over 2000bp. The barley cv Tweed cDNA library was constructed using a mixture of oligo-dT and random primers, which can reduce the average insert size due to random-priming near the 5' end of the poly A<sup>+</sup> RNA, although this cannot explain the short length of oligo-dT-primed and possibly random-primed partial 3' nitrite reductase cDNA clones. The poor quality of the cDNA library was illustrated when a potato cv Desireé leaf cDNA library (Kossmann *et al*, 1992) was screened for comparative purposes using the tobacco leaf nitrite reductase cDNA clone *nir-3* (Kronenberger *et al*, 1993) as a heterologous probe and thirteen hybridising potato cDNA clones were isolated, all of which were, or were close to, full length.

Partial sequencing of the largest putative barley nitrite reductase cDNA clone, HvNiR61 (1343bp), and subsequent EMBL database searches showed high homology of the HvNiR61 cDNA sequence to the barley cv Maris Mink nitrite reductase (Ward *et al*, 1995) (100%), maize nitrite reductase (Lahners *et al*, 1988) (89%) and rice nitrite reductase (Terada *et al*, 1995) (89%) cDNA sequences, thus confirming the identity of this HvNiR61 as a nitrite reductase cDNA clone. As anticipated for a barley nitrite

reductase cDNA clone, northern analysis of barley cv Tweed leaf tissue demonstrated hybridisation of HvNiR61 to a nitrate-induced transcript of 2300bp and further work should involve the use of this clone to rescreen the barley cv Tweed cDNA library in order to isolate a full-length or a random-primed clone which covers the 5' end of the barley nitrite reductase cDNA sequence and overlaps with HvNiR61.

Sequencing of the 5' ends of the two largest putative potato leaf nitrite reductase cDNA clones, StNiR12 and StNiR14, and subsequent EMBL database searches revealed that StNiR12 and StNiR14 possessed high nucleotide sequence homologies to the same 5' untranslated region of the tobacco leaf nitrite reductase cDNA clone *nir-1* (Vaucheret *et al*, 1992a), being 86% for StNiR12 and 73% for StNiR14. However, a nucleotide sequence comparison between the 5' ends of StNiR12 and StNiR14 revealed differences in the two sequences, suggesting that potato expresses at least two different nitrite reductase genes in the leaf.

## 7.7 Environmental control of nitrite reductase in barley

Increases in *in vitro* methyl viologen nitrite reductase activity in response to nitrate and light is due to *de novo* synthesis of nitrite reductase apoprotein transcript, with concomitant increases in nitrite reductase cross-reacting material and nitrite reductase activity, and is not the result of some form of inactivation/activation mechanism of pre-existing enzyme molecules. Thus, regulation of nitrite reductase in barley leaf is similar to that reported in leaf tissue of wheat (Small and Gray, 1984) and pea (Gupta and Beevers, 1984) where increases in nitrite reductase activity correlated with increases in nitrite reductase cross-reacting material. Nitrite reductase transcript also shows a strong correlation with NiR-CRM and nitrite reductase activity and both nitrate and light are required for high levels of nitrite

reductase transcript. Thus, it appears that in barley, nitrate and light act predominantly at the transcriptional level as in spinach (Back *et al*, 1988), maize (Bowsher *et al*, 1991; Lahners *et al*, 1988), tobacco (Faure *et al*, 1991) and birch (Friemann *et al*, 1992b). These results suggest that nitrate and light modulate the synthesis of leaf nitrite reductase mRNA.

## 7.8 Future work

Further analysis of mutant lines should allow a fuller characterisation of the *nir1* mutation(s) at the molecular level.

Rescreening the barley cv Tweed cDNA library with the 1.35kb partial barley nitrite reductase cDNA clone HvNiR61 should allow isolation of an overlapping barley nitrite reductase cDNA covering the 5' region of the barley nitrite reductase (*Nii*) cDNA sequence. These clones could then be sequenced, and the sequences used to design primers for RT-PCR amplification of *Nii* cDNA from poly A<sup>+</sup> RNA isolated from the *nir1* mutant STA3999. This RT-PCR product could then be sequenced and compared to the wild-type Tweed *Nii* cDNA sequence to confirm the identity of the *Nir1* locus as *Nii*, and to establish the nature of the *nir1* mutation in STA3999.

Transgenic techniques would allow the study of *nir1* mutants expressing an *Nii* sense construct to determine whether nitrite reductase activity can be restored in these mutants.

Study of the regulation of nitrate reductase and nitrite reductase gene expression in the *nir1* mutants and wild-type cultivars of barley under different light and nitrogen metabolite conditions would allow further characterisation of the regulation of nitrate assimilation in barley. Isolation of an anti-barley nitrate reductase antibody would greatly aid the characterisation of the regulation of nitrate assimilation in barley.

Further biochemical studies could also be performed to characterise the effect of the *nir1* mutation on other biochemical pathways, such as sulphate assimilation and the GS-GOGAT pathway, and study effects on amino acid biosynthesis.

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## APPENDIX



## A.1. COMMONLY USED BUFFERS AND SOLUTIONS

### 50x TAE Buffer

2M Tris  
5.71% (v/v) Glacial acetic acid  
50mM Na<sub>2</sub>EDTA

Adjust to pH 8.0 with 10N sodium hydroxide, then autoclave.

### 10x TBE Buffer

890mM Tris  
890mM Boric acid  
20mM Na<sub>2</sub>EDTA

Adjust to pH 8.3 with 10N sodium hydroxide, then autoclave.

### TE Buffer

10mM Tris  
1mM Na<sub>2</sub>EDTA

Adjust to pH 8.0 with 10N sodium hydrochloride, then autoclave.

### 20x SSC Buffer

3M Sodium chloride  
300mM Tri-sodium citrate

Adjust to pH 7.0 with 10N sodium hydroxide.

### **20x SSPE Buffer**

3M Sodium chloride  
200mM Sodium dihydrogen phosphate  
20mM Na<sub>2</sub>EDTA

Adjust to pH 7.4 with 10N sodium hydroxide.

### **10x Electrophoresis Loading Buffer**

50% (v/v) Glycerol  
0.25% (w/v) Bromophenol blue

In 1x TAE.

Autoclave.

### **DEPC-treated sterile distilled water**

0.1% DEPC

Stir for three hours, then autoclave.

## A.2. MICROBIOLOGICAL MEDIA

### LB Broth

1% (w/v) Sodium chloride

1% (w/v) Bactotryptone

0.5% (w/v) Yeast extract

Adjust pH to 7.5 with 10M sodium hydroxide, then autoclave.

### NZY Broth

0.5% (w/v) Sodium chloride

0.2% (w/v) Magnesium sulphate (7-hydrate)

0.5% (w/v) Yeast extract

1% (w/v) NZ Amine A (casein hydrolysate)

Adjust to pH 7.5 with 10M sodium hydroxide, then autoclave.

### LB Agar and NZY Agar

As for broth, but with the addition of 1.5% (w/v) bacteriological agar.

### Top Agarose

As for NZY broth, but with the addition of 0.7% (w/v) agarose.

### Antibiotics

Filter sterilise using a 0.22 $\mu$ m filter (Millipore, UK).

Ampicillin (sodium salt):

*Stock*, 50mgml<sup>-1</sup> in water

*Working*, 50 $\mu$ gml<sup>-1</sup>

Tetracycline (hydrochloride):	<i>Stock</i> , 5mgml <sup>-1</sup> in 50% (v/v) ethanol <i>Working</i> , 12.5µgml <sup>-1</sup>
Kanamycin:	<i>Stock</i> , 50mgml <sup>-1</sup> in water <i>Working</i> , 50µgml <sup>-1</sup>

### A.3. BACTERIAL STRAINS

<i>E. coli</i>	DH5a	Growth medium: LB Antibiotic: none
<i>E. coli</i>	XL-1 Blue	Growth medium: LB Antibiotic: Tetracycline
<i>E. coli</i>	SOLR	Growth medium: LB Antibiotic: Kanamycin